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(54) Title: MUTANT PNEUMOLYSIN PROTEINS

(57) Abstract: The invention relates to immunogenic compositions comprising mutant *Streptococcus pneumoniae* pneumolysin proteins. The invention further relates to such proteins and nucleic acids encoding these proteins. In particular embodiments, the invention is directed to an isolated mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein. In particular embodiments, the mutant PLY protein differs from the wild type protein by the substitution or deletion of amino acids within this region, including the deletion of two adjacent amino acids within the region of amino acids 144 to 151 of the wild type sequence.



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MUTANT PNEUMOLYSIN PROTEINSField of the Invention

The present invention relates to immunogenic compositions
5 comprising mutant *Streptococcus pneumoniae* pneumolysin
proteins. The invention further relates to such proteins and
nucleic acids encoding these proteins.

Background of the Invention

10 *Streptococcus pneumoniae* is an important pathogen,
causing invasive diseases such as pneumonia, meningitis and
bacteraemia. Even in regions where effective antibiotic
therapy is freely available, the mortality rate from
pneumococcal pneumonia can be as high as 19% in hospitalised
15 patients. In developing countries, in excess of 3 million
children under the age of 5 years die each year from
pneumonia, of which *S. pneumoniae* is the commonest causative
agent. *S. pneumoniae* also causes less serious, but highly
prevalent infections such as otitis media and sinusitis, which
20 have a significant impact on health-care costs in developed
countries. Otitis media is especially important in young
children, while sinusitis affects both children and adults.

The heptavalent polysaccharide conjugate vaccine from
Wyeth, sold as Prevnar® in the United States and as Prevenar®
25 in the rest of the world, is currently the only effective
conjugate vaccine available for protection against
Streptococcus pneumoniae infection (Kyaw et al, 2002;
Hausdorff et al, 2000). The vaccine comprises seven purified
Streptococcus capsular polysaccharides (serotypes 4, 6B, 9V,
30 14, 18C, 19F and 23F) out of a possible 90 (Kalin, 1998), each
conjugated to a carrier protein. Preparation of such a vaccine
is described in US Patent 4,673,574 (Anderson). The protein
used for conjugation of the capsular polysaccharides is a
diphtheria toxoid, CRM₁₉₇, offering an increase in the
35 immunogenicity of the vaccine in infants (Blum et al, 2000;
Katkocin, 2000). However, each serotype of *S. pneumoniae* has a

structurally distinct capsular polysaccharide, such that immunization with one serotype tends not to confer protection against the majority of the other serotypes, although some cross-protection does occur against vaccine-related serotypes
5 (Whitney et al., 2003).

Complementary approaches to serotype-specific immunization are being investigated. A possibility is to also use a species-common virulence factor such as Pneumolysin (PLY), the 53kDa toxin produced by all invasive strains of *S. pneumoniae* (Paton et al, 1993). PLY could be used alone or as
10 a carrier protein conjugated to the polysaccharides in Prevnar®, offering increased efficacy. Alexander et al (1994) demonstrated that immunisation of mice with a PLY toxoid conferred immune protection upon challenge with 9 different
15 serotypes of *S. pneumoniae*. PLY has been shown to stimulate an immune response similar to that of *S. pneumoniae* infection by activating the classical complement pathway (Paton et al, 1984) and inducing apoptosis of neutrophils and macrophages (Cockeran et al, 2002; Kadioglu et al, 2000).

PLY belongs to the group of Cholesterol-binding Cytolysins (CBCs) that bind to the cholesterol of host cell membranes prior to formation of large 30-50mer ring structures that create lytic pores (Palmer, 2001; Jedrzejewski, 2001). The mechanism of pore-formation is not fully understood and there
20 is much debate over the sequence of events (Bonev et al, 2000; Shepard et al, 1998). However, the ability to form pores means that native PLY is highly toxic, which is a problem in terms of the development of immunogenic compositions.

Although the conjugation process used in production would
30 render PLY non-toxic, it would be more favourable to start with a non-toxic form. Further, a toxic form would be difficult to use in preparation of unconjugated immunogenic compositions. The toxicity of PLY can be significantly reduced by site-directed mutagenesis to create PLY toxoids, known as
35 Pneumolysoids (Paton, 1996).

A variety of such toxoids exist and have been shown to give immune protection, either independently or when conjugated to polysaccharides, to mice in response to a challenge with virulent type 2 D39 *S. pneumoniae* (Paton et al, 1991; Alexander et al, 1994). Most mutations have previously been created in the highly conserved 11 amino acid region near the C' terminus (Mitchell et al, 1992; Berry et al, 1995). This site has been shown to be involved in binding to the host cell (de los Toyos et al, 1996). A number of such mutated forms of PLY are described in International Patent Application WO 90/06951; each of the mutations described in this publication is towards the C' terminus of the protein.

A further problem with PLY is that it aggregates upon large-scale production, a problem which must be solved in order for PLY to be used in immunogenic compositions. It is believed that the aggregation of PLY is related to the oligomerisation of PLY involved in pore formation. The present invention thus attempts to reduce or eliminate PLY-PLY interaction (oligomerisation), such that the chance of aggregation during large-scale production will decrease, thereby creating an easily purified form of PLY.

Toyos et al (1996) describe the raising of monoclonal antibodies (mAbs) to various regions of PLY, and probing of the whole toxin and a 'proteinase K nicked' form. Proteinase K cuts PLY into a 37kDa and 15kDa fragment. Antibody mAb PLY 4 only recognised whole PLY, and neither of the fragments, indicating that the epitope on PLY for this mAb is within the nicked region. When PLY was pre-incubated with mAb PLY 4, then added to liposomes, the toxin no longer formed pores on the liposome membrane. This implies that the site blocked by mAb PLY 4 (thought to be the Asparagine N₁₄₃ region) is the site responsible for interaction with other PLY monomers to form oligomeric pores. Oligomerisation of Streptolysin O from *Streptococcus sp.* can also be blocked by mAbs as demonstrated by Hugo et al in 1986. It is unknown whether the antibodies directly prevent oligomerisation by binding to the

oligomerisation site or if there is an association that sterically hinders the interaction of toxin monomers.

Monoclonal antibody PLY 4 has been further characterised by Suárez-Álvarez et al (2003) and they suggest that the epitope for mAb PLY 4 is further downstream than the N₁₄₃ region initially proposed by Toyos et al in 1996. The site of recognition now appears to be conformation dependent and within amino acids E₁₅₁ - Y₂₄₇ and not within the N₁₄₃ region.

Previously a N₁₄₂N₁₄₃ deletion and N₁₄₃D substitution within PLY were created by the present inventors as initial steps to understanding this region and its role in oligomerisation. Characterisation of both mutants revealed identical behaviour to native PLY in terms of haemolysis and pore formation (Search, 2002), suggesting that oligomerisation was not blocked, and the toxicity of the mutants remained unchanged. Thus, previously created mutant PLY forms do not exhibit reduced toxicity or reduced oligomerisation, suggesting that these mutations will not be of assistance in the production of immunogenic compositions.

20

Summary of the Invention

The present invention relates broadly to immunogenic compositions comprising mutant *Streptococcus pneumoniae* pneumolysin proteins. The invention further relates to such proteins and nucleic acids encoding these proteins.

Thus, in one aspect, the invention is directed to an isolated mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein. The mutation may be located within the region of amino acids 144 to 151 of the wild type sequence.

The mutation may be a deletion or substitution of one or more amino acids within the region of amino acids 144 to 161 of the wild type sequence.

The mutant PLY protein may differ from the wild type protein by the substitution or deletion of one or more amino acids within the region of amino acids 144 to 151 of the wild type sequence.

5 For example, the mutant PLY protein may differ from the wild type protein by the substitution or deletion of two adjacent amino acids within the region of amino acids 144 to 151 of the wild type sequence, as exemplified by the deletion of amino acids valine 144 and proline 145, alanine 146 and
10 arginine 147, methionine 148 and glutamine 149, or tyrosine 150 and glutamic acid 151.

Any of the foregoing mutant PLY proteins may further comprise at least one amino acid substitution or deletion in at least one of the regions of amino acids 257-297, 367-397 or
15 424-437 of the wild type sequence.

The isolated mutant PLY protein has reduced toxicity to mammals. This is typically a consequence of having reduced pore-forming activity, which may be associated with reduced haemolytic activity and/or reduced oligomerisation activity,
20 as compared with wild type PLY protein. Desirably, although not necessarily, the mutant PLY protein has reduced oligomerisation activity to facilitate purification and subsequent manipulation.

In a further aspect, the invention is directed to an
25 immunogenic conjugate comprising: (a) a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein; and (b) an isolated mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of
30 amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein. The mutation may be located within the region of amino acids 144 to 151 of the wild type sequence.

The mutation may be a substitution or deletion of one or
35 more amino acids within the region of amino acids 144 to 161 of the wild type sequence.

The mutant PLY protein of the immunogenic conjugate may differ from the wild type protein by the substitution or deletion of one or more amino acids within the region of amino acids 144 to 151 of the wild type sequence.

5 For example, the mutant PLY protein of the immunogenic conjugate differs from the wild type protein by the substitution or deletion of two adjacent amino acids within the region of amino acids 144 to 151 of the wild type sequence, as exemplified by the deletion of amino acids valine
10 144 and proline 145, alanine 146 and arginine 147, methionine 148 and glutamine 149, or tyrosine 150 and glutamic acid 151.

Any of the foregoing mutant PLY proteins of the immunogenic conjugate may further comprise at least one amino acid substitution or deletion in at least one of the regions
15 of amino acids 257-297, 367-397 or 424-437 of the wild type sequence.

The saccharide, oligosaccharide or polysaccharide of the immunogenic conjugate may be derived from *Streptococcus pneumoniae*.

20 In a further aspect, the invention provides an isolated and purified nucleic acid sequence comprising a nucleic acid sequence a) encoding a mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of
25 amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein; or b) which is complementary to a nucleic acid sequence defined in a).

The mutation may be located within the region of amino
30 acids 144 to 151 of the wild type sequence.

The mutation may be a substitution or deletion of one or more amino acids within the region of amino acids 144 to 161 of the wild type sequence.

The nucleic acid sequence may encode a mutant PLY protein
35 which differs from the wild type protein by the substitution

or deletion of one or more amino acids within the region of amino acids 144 to 151 of the wild type sequence.

For example, the nucleic acid sequence may encode a mutant PLY protein which differs from the wild type protein by the substitution or deletion of two adjacent amino acids within the region of amino acids 144 to 151 of the wild type sequence, as exemplified by the substitution or deletion of amino acids valine 144 and proline 145, alanine 146 and arginine 147, methionine 148 and glutamine 149, or tyrosine 150 and glutamic acid 151.

The mutant PLY proteins encoded by the nucleic acid sequence may be any of the foregoing, and may further comprise at least one amino acid substitution or deletion in at least one of the regions of amino acids 257-297, 367-397 or 424-437 of the wild type sequence.

In a still further aspect, the invention provides a recombinant expression vector which comprises any of the foregoing isolated and purified nucleic acid sequences encoding a mutant PLY protein, as well as a recombinant host cell transformed, transfected or infected with such a recombinant expression vector.

In another aspect, the invention provides a method of producing an isolated mutant pneumolysin (PLY) protein of the invention, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein, the method comprising: a) transforming, transfecting or infecting a host cell with a recombinant expression vector as described above and culturing the host cell under conditions which permit the expression of said mutant PLY protein by the host cell; and b) recovering the mutant PLY protein from the culture.

In still another aspect, there is provided an immunogenic composition which comprises: a) an isolated mutant pneumolysin (PLY) protein, in unconjugated form or as part of

an immunogenic conjugate as described above, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein; and b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

The mutation may be located within the region of amino acids 144 to 151 of the wild type sequence.

10 The mutation may be a substitution or deletion of one or more amino acids within the region of amino acids 144 to 161 of the wild type sequence.

The isolated mutant pneumolysin (PLY) protein of the composition may differ from the wild type PLY protein by the substitution or deletion of one or more amino acids within the region of amino acids 144 to 151 of the wild type sequence.

For example, the mutant PLY protein may differ from the wild type PLY protein by the substitution or deletion of two adjacent amino acids within the region of amino acids 144 to 151 of the wild type sequence, as exemplified by the substitution or deletion of amino acids valine 144 and proline 145, alanine 146 and arginine 147, methionine 148 and glutamine 149, or tyrosine 150 and glutamic acid 151.

The immunogenic composition may contain any of the foregoing mutant PLY proteins, in unconjugated form or as part of an immunogenic conjugate as described above, which further comprises at least one amino acid substitution or deletion in at least one of the regions of amino acids 257-297, 367-397 or 424-437 of the wild type sequence.

30 Thus, the immunogenic composition may comprise: a) an immunogenic conjugate comprising: (i) a saccharide, oligosaccharide or polysaccharide derived from *Streptococcus*, e.g. *Streptococcus pneumoniae*; and (ii) an isolated mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the

wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein; and b) one or more of a physiologically acceptable adjuvant, diluent or carrier. The composition may comprise a plurality of
5 *Streptococcus pneumoniae* subtypes of saccharides, oligosaccharides or polysaccharides.

In further aspects, the invention is directed to a method of prophylaxis for a mammal, the method comprising the step of administering to a subject mammal an immunogenic composition
10 which comprises: a) an isolated mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to
15 that of the wild-type protein; and b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

The immunogenic composition may comprise: a) an immunogenic conjugate comprising: (i) a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or
20 protein; and (ii) an isolated mutant pneumolysin (PLY) protein as described herein.

In another aspect, the invention is directed to the use of any of the isolated mutant PLY proteins or immunogenic conjugates of the invention in the preparation of an
25 immunogenic composition. The invention also provides a method of preparing an immunogenic composition, comprising the step of admixing a mutant protein or immunogenic conjugate of the invention with a pharmaceutically acceptable carrier.

The immunogenic compositions of the invention may be used
30 for the prophylaxis or treatment of bacterial infection. In particular they are useful for the prophylaxis or treatment of infection by bacteria having cholesterol-binding cytolysins (see e.g. Palmer, 2001) which are immunologically cross reactive with PLY; that is to say, which are capable of being
35 bound by antibodies which will bind to PLY. Preferably

though, the bacteria are *Streptococci*, preferably *Streptococcus pneumoniae*.

In a further aspect, the present invention provides an isolated mutant PLY protein or immunogenic conjugate of the invention for use in a method of medical treatment.

In a still further aspect, the invention is directed to a method of preparation of an immunogenic composition, the method comprising the steps of: providing an isolated mutant PLY protein as described herein; and conjugating the mutant protein to a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein. The mutant protein in the foregoing method may be conjugated to a *Streptococcus pneumoniae* polysaccharide. The method may comprise the further step of admixing the conjugate thus obtained with a pharmaceutically acceptable carrier.

In a further aspect, the invention is directed to a method of screening candidate mutant PLY proteins for suitability for use in immunogenic compositions, the method comprising the steps of: providing a mutant PLY protein; testing the mutant protein for haemolytic activity; testing the mutant PLY protein for oligomerisation activity; and comparing the mutant PLY protein haemolytic and oligomerisation activity with those of a non-mutant protein.

Brief Description of the Figures

Figure 1 shows the amino acid sequence of wild-type pneumolysin;

Figure 2 shows a western blot of PLY deletion mutants detected by mAb PLY4;

Figure 3 shows the results of a quantitative haemolytic assay comparing WT PLY to $\Delta 6$ PLY mutant;

Figure 4 shows the results of a cytotoxicity assay comparing WT PLY to $\Delta 6$ PLY mutant;

Figure 5 shows electron micrographs of WT PLY treated erythrocyte membranes;

Figure 6 shows IL-6 levels in lung tissue after treatment with WT PLY or $\Delta 6$ PLY;

Figure 7 shows IL-6 levels in lung lavage after treatment with WT PLY or $\Delta 6$ PLY;

5 Figure 8 shows total protein levels in bronchoalveolar lavage after treatment with WT PLY or $\Delta 6$ PLY;

Figure 9 shows anti-PLY antibody levels in response to immunization of mice with WT PLY or $\Delta 6$ PLY;

10 Figure 10 shows the degree of haemolysis in relation to toxin concentration in SRBC (sheep red blood cell) treated with WT PLY or the deletion mutant $\Delta A146$ PLY.

Figure 11 compares the haemolytic activity of WT PLY and the mutants PLY W433F, $\Delta 6$ PLY, $\Delta 7$ PLY, $\Delta 8$ PLY and $\Delta A146$ PLY;

15 Figure 12 shows the cytotoxicity to murine L929 fibroblasts of WT PLY and the mutants PLY W433F, $\Delta 6$ PLY, $\Delta 7$ PLY, $\Delta 8$ PLY and $\Delta A146$ PLY;

Figure 13 shows that $\Delta A146$ PLY does not cause degranulation of RBL-2H3 mast cells, while WT PLY does;

20 Figure 14 shows analysis of core body temperature following treatment with wild type PLY or $\Delta A146$ PLY.

Detailed Description of the Invention

PLY is a member of the group of cholesterol-binding cytolysins (CBCs). The amino acid sequence of wild type pneumolysin is given in Figure 1. Figure 1 also indicates the
25 GenBank identification number of the sequence, derived from NCBI-GenBank Flat File Release 141.0, April 15 2004.

The present invention relies on the identification of a number of PLY forms having a mutation within the region of
30 amino acids 144 to 161 of the wild type PLY protein which have reduced toxicity, as reflected by a reduction in haemolytic activity and/or oligomerisation. The consensus sequence of this region is as follows: VPARMQYEKITAHSMEQL (see Figure 1).

In one aspect, the present invention relates to mutant
35 PLY proteins which differ from the wild type protein by the

mutation within the region of amino acids 144 to 151 of the wild type sequence. The consensus sequence of this region is as follows: VPARMQYE (see Figure 1).

5 The mutant may have a substitution or deletion of one or more amino acids within the region of amino acids 144 to 161, e.g. 144 to 151.

Thus, in all aspects of the invention, the mutant pneumolysin may have a mutation, e.g. a substitution or deletion, at one or more of amino acids 144, 145, 146, 147, 10 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160 or 161 of the wild type sequence.

The invention further relates to mutant PLY proteins which differ from the wild type protein by the substitution or deletion of two adjacent amino acids within the region of 15 amino acids 144 to 151 of the wild type sequence. Examples of such double mutants are those which contain substitutions or deletions of amino acids valine 144 and proline 145, alanine 146 and arginine 147, methionine 148 and glutamine 149, or tyrosine 150 and glutamic acid 151.

20 These mutant PLY proteins are used *per se* in immunogenic compositions, together with one or more of a physiologically acceptable adjuvant, diluent or carrier.

Alternatively, these mutant PLY proteins are conjugated to a saccharide, oligosaccharide, polysaccharide, peptide, 25 polypeptide or protein, from the same or heterologous organism, to form conjugates which are used in immunogenic compositions, together with one or more of a physiologically acceptable adjuvant, diluent or carrier. Thus the saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or 30 protein to which the mutant PLY is conjugated may be from *Streptococcus*, e.g. *Streptococcus pneumoniae*. They may be derived from the bacterial capsule.

In either unconjugated or conjugated form, the mutant PLY proteins contained in immunogenic compositions are used in 35 prophylaxis or therapy.

In either unconjugated or conjugated form, the mutant PLY proteins may further contain at least one amino acid substitution or deletion in at least one of the regions of amino acids 257-297, 367-397 or 424-437 of the wild type sequence. These further substitutions or deletions are described in Paton et al. published International Patent Application WO 90/06951.

According to a further aspect of the present invention, there is provided an immunogenic composition comprising an isolated mutant PLY protein as described herein. In one embodiment, the immunogenic composition is a *Streptococcus pneumoniae* immunogenic composition.

The mutant PLY proteins may retain immunogenic activity in mammals. By "immunogenic in mammals" is meant that mammalian immune systems will produce antibodies to the mutant PLY protein, and that these antibodies will also recognise wild type PLY protein. Similarly, mammalian antibodies to the wild type PLY protein will also recognise the mutant PLY protein. Preferably the mutant protein is immunogenic in humans. Preferably the mutant PLY protein will stimulate the mammalian immune system to produce antibodies which bind to the wild type VPARMQYEKITAHSMEQL or VPARMQYE sequence.

In one embodiment, the mutation is in the region of the PLY protein involved in oligomerisation of the wild type protein.

Without being bound by theory, it is believed that the mutant PLY protein has reduced toxicity as a result of reduced pore-formation activity compared with wild type PLY protein. This is believed to be associated with reduced oligomerisation activity and/or reduced haemolytic activity. Toxicity may be measured directly. Alternatively, one or more of pore formation, oligomerisation and haemolysis may be measured to provide an indication of likely toxicity.

Deletions and substitutions are examples of mutations which may be used to provide the PLY mutant proteins with reduced toxicity. Non-conservative substitutions may be

particularly suitable for reducing toxicity of the PLY mutant, as a mutant having a non-conservative mutation is less likely to retain wild-type levels of function than one having a conservative substitution.

5 A conservative substitution may be defined as a substitution within an amino acid class and/or a substitution that scores positive in the BLOSUM62 matrix as shown below, thus a non-conservative substitution may be defined as a substitution between amino acid classes, or which does not
10 score positive in the BLOSUM62 matrix.

According to one classification, the amino acid classes are acidic, basic, uncharged polar and nonpolar, wherein acidic amino acids are Asp and Glu; basic amino acids are Arg, Lys and His; uncharged polar amino acids are Asn, Gln, Ser,
15 Thr and Tyr; and non-polar amino acids are Ala, Gly, Val, Leu, Ile, Pro, Phe, Met, Trp and Cys.

According to another classification, the amino acid classes are small hydrophilic, acid/acidamide/hydrophilic, basic, small hydrophobic and aromatic, wherein small
20 hydrophilic amino acids are Ser, Thr, Pro, Ala and Gly; acid/acidamide/hydrophilic amino acids are Asn, Asp, Glu and Gln; basic amino acids are His, Arg and Lys; small hydrophobic amino acids are Met, Ile, Leu and Val; and aromatic amino acids are Phe, Tyr and Trp.

25 Conservative substitutions, which score positive in the BLOSUM62 matrix, are as follows:

Original Residue	C	S	T	P	A	G	N	D	E	Q	H	R	K	M	I	L	V	F	Y	W
Substitution	-	T A N	S	-	S	-	S D H	N E	D Q K	E R K	N Y	Q K	E Q R	I L V	M L V	M I V	M I L	Y W	H F W	F Y

The mutant pneumolysin protein of the invention
30 preferably has at least 80% amino acid identity with the wild type sequence as shown in Figure 1. The mutant may have at

least 85% identity, at least 90% identity, or at least 95% identity with the wild type sequence.

Percent (%) amino acid sequence identity with respect to a reference sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. % identity values may be determined by WU-BLAST-2 (Altschul et al., Methods in Enzymology, 266:460-480 (1996)). WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span = 1, overlap fraction = 0.125, word threshold (T) = 11. A % amino acid sequence identity value is determined by the number of matching identical residues as determined by WU-BLAST-2, divided by the total number of residues of the reference sequence (gaps introduced by WU-BLAST-2 into the reference sequence to maximize the alignment score being ignored), multiplied by 100.

Percent (%) amino acid similarity is defined in the same way as identity, with the exception that residues scoring a positive value in the BLOSUM62 matrix are counted. Thus, residues which are non-identical but which have similar properties (e.g. as a result of conservative substitutions) are also counted.

Amino acid insertions within the region of amino acids 144 to 161, e.g. 144 to 151, may also be used to reduce toxicity of the PLY mutant. For example, insertions of 1, 2, 3, 4, 5, 10, 15, 20 or more amino acids may be used. However, deletions and substitutions are generally preferred to insertions as they are less likely to disrupt the wild type epitope; such disruption could reduce the immunogenicity of the mutant protein, which may be undesirable in an immunogenic composition.

In another aspect of the present invention, the mutant
PLY protein is conjugated to a saccharide, oligosaccharide,
polysaccharide, peptide, polypeptide or protein to form an
immunogenic conjugate. In this aspect, the mutant PLY protein
5 may retain its immunogenicity, or that immunogenicity may be
ablated. In either event, the mutant PLY protein serves to
enhance the immunogenicity of the saccharide, oligosaccharide,
polysaccharide, peptide, polypeptide or protein in the
conjugate.

10 Such saccharides, oligosaccharides, polysaccharides,
peptides, polypeptides or proteins are each conjugated to the
mutant PLY protein in any suitable manner, including, but not
limited to: (1) direct coupling via protein functional groups
(e.g., thiol-thiol linkage, amine-carboxyl linkage, amine-
15 aldehyde linkage; enzyme direct coupling); (2)
homobifunctional coupling of amines (e.g., using bis-
aldehydes); (3) homobifunctional coupling of thiols (e.g.,
using bis-maleimides); (4) homobifunctional coupling via
photoactivated reagents (5) heterobifunctional coupling of
20 amines to thiols (e.g., using maleimides); (6)
heterobifunctional coupling via photoactivated reagents (e.g.,
the β -carbonyldiazo family); (7) introducing amine-reactive
groups into a poly- or oligosaccharide via cyanogen bromide
activation or carboxymethylation; (8) introducing thiol-
25 reactive groups into a poly- or oligosaccharide via a
heterobifunctional compound such as maleimido-hydrazide; (9)
protein-lipid conjugation via introducing a hydrophobic group
into the protein and (10) protein-lipid conjugation via
incorporating a reactive group into the lipid. Also,
30 contemplated are heterobifunctional "non-covalent coupling"
techniques such the Biotin-Avidin interaction. For a
comprehensive review of conjugation techniques, see Aslam and
Dent (1998), incorporated hereinafter by reference in its
entirety.

35 Further methods of conjugating a peptide, polypeptide or
protein to a protein are described in U.S. provisional patent

applications 60/530,480 and 60/530,481, both filed December 17, 2003, and both incorporated by reference in their entirety.

5 Additionally, US Patent 5,565,204 to Kuo et al. described a method for conjugating such polysaccharides to the wild type PLY protein; that method is also suitable for conjugating such polysaccharides to the mutant PLY proteins of this invention.

The immunogenic compositions of the present invention may be conjugated immunogenic compositions. Each immunogenic
10 composition may comprise one or more saccharides, oligosaccharides, polysaccharides, peptides, polypeptides or proteins, which may be derived from the source organism (*S. pneumoniae*) of the wild type PLY protein. In non-limiting examples, such components may be derived from the capsule of
15 the organism.

In one embodiment, the saccharides, oligosaccharides or polysaccharides are derived from more than one serotype of *S. pneumoniae*; the particular serotypes will depend on the intended use for the immunogenic composition and the
20 prevalence of these serotypes in the target population.

Alternatively, the saccharides, oligosaccharides, polysaccharides, peptides, polypeptides or proteins are derived from a heterologous organism (that is, an organism other than *S. pneumoniae*). In the case of saccharides,
25 oligosaccharides or polysaccharides, multiple serotypes may be obtained from, without limitation, *Neisseria meningitidis* (for example, from serotypes A, C, Y and W135), *Staphylococcus aureus* and *Haemophilus influenzae*.

In certain aspects of the invention, the mutant PLY
30 protein is conjugated to another peptide, polypeptide or protein of *S. pneumoniae*. Alternatively, the mutant PLY protein is conjugated to a peptide, polypeptide or protein from a heterologous organism, including a human. For example, the mutant PLY protein is conjugated to another peptide,
35 polypeptide or protein, which is from a pathogenic virus, bacterium, fungus or parasite, or (2) from a cancer cell or

tumor cell, or (3) from an allergen so as to interfere with the production of IgE so as to moderate allergic responses to the allergen, or (4) from amyloid precursor protein (APP) so as to prevent or treat disease characterized by amyloid
5 deposition in a vertebrate host.

The moiety of APP which is conjugated to the mutant PLY protein may be the β -amyloid peptide (also referred to as A β peptide), which is an internal, 39-43 amino acid fragment of (APP), which is generated by processing of APP by the β and
10 γ secretase enzymes. An example of such a peptide is the A β 1-42 peptide, which has the following amino acid sequence:
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His
Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala

15 The A β component may be further administered in the form of a fragment conjugated to the mutant PLY protein. Non-limiting examples of such fragments include A β 1-3, 1-4, 1-5, 1-6, 1-7, 3-7, 3-8, 3-9, 3-10, 3-11, 1-10 and 1-12. The use of A β and fragments thereof, as conjugated to proteins other
20 than PLY, is described in published International Patent Applications WO 99/27944 and WO 00/72880, which are hereby incorporated by reference.

A further aspect of the present invention provides a method of prophylaxis or treatment for a mammal, the method
25 comprising the step of administering to a subject mammal an immunogenic composition comprising an isolated PLY protein having a mutation as described herein, where the mutant PLY protein is unconjugated or conjugated as described herein. Typically the method is intended for prophylaxis or treatment
30 of infection by one or more species or strains of bacteria having a cholesterol-binding cytolysin which is immunologically cross-reactive with wild-type pneumolysin, particularly *Streptococcus*, and in particular, *Streptococcus pneumoniae*. Other species, and their cytolysins, include
35 *Clostridium perfringens* (Perfringolysin O), *Streptococcus*

intermedius (Intermedilysin), *Bacillus alvei* (Alveolysin),
Bacillus anthracis (Anthrolysin), *Bacillus cereus*
(Cereolysin), *Listeria ivanovii* (Ivanolysin O), *Clostridium*
novyi (Novyilysin), *Arcanobacterium pyogenes* (Pyolysin),
5 *Listeria seeligeri* (Seeligeriolysin O), *Clostridium septicum*
(Septicolysin), *S. pyogenes* (Streptolysin O), *Streptococcus*
suis (Suilysin), *Clostridium tetani* (Tetanolysin) *Listeria*
monocytogenes (Listeriolysin O), *Streptococcus equisimilis*
(Streptolysin O), *S. canis* (Streptolysin O), *Bacillus*
10 *thuringiensis* (Thuringiolysin O), *B. laterosporus*
(Laterosporolysin O), *Clostridium botulinum* (Botulinolysin), *C.*
chauvoei (Chauveolysin), *C. bifermentans* (Bifermentolysin),
C. sordellii (Sordellilysin) (see e.g. Palmer 2001).

The mode of administration of an immunogenic composition
15 of the invention, whether of the mutant PLY protein alone or
as part of an immunogenic conjugate, may be by any suitable
route which delivers an immunoprotective amount of the protein
to the subject. One such route is the parenteral route, such
as by intramuscular or subcutaneous administration. Other
20 modes of administration may also be employed, where desired,
such as the mucosal route, such as by oral, rectal, buccal or
intranasal administration, or via other parenteral routes,
i.e., intradermally or intravenously.

Generally, the immunogenic composition will usually be
25 presented as a pharmaceutical formulation including a
physiologically acceptable carrier or excipient, for example,
sterile water or sterile isotonic saline, as well as any and
all solvents, dispersion media, coatings, antibacterial and
antifungal agents, isotonic and absorption delaying agents,
30 and the like, compatible with administration to humans. The
appropriate carrier will be evident to those skilled in the
art and will depend in large part upon the route of
administration. The immunogenic composition of the present
invention may also include a physiologically acceptable
35 diluent such as sterile water or sterile isotonic saline. The
formulation may be prepared by conventional means.

It will be understood, however, that the specific dose level for any particular recipient mammal will depend upon a variety of factors including age, general health, and sex; the time of administration; the route of administration; synergistic effects with any other drugs being administered; and the degree of protection being sought. Of course, the administration can be repeated at suitable intervals if necessary.

The mammal may be a human, or may be a non-human mammal. The immunogenic composition may be administered in any convenient manner; for example, those described above.

The immunogenic composition of the present invention may include one or more physiologically acceptable adjuvants. A substance that enhances the immune response when administered together with an immunogen or antigen is known as an adjuvant. A number of cytokines or lymphokines have been shown to have immune modulating activity, and thus may be used as adjuvants, including, but not limited to, the interleukins 1- α , 1- β , 2, 4, 5, 6, 7, 8, 10, 12 (see, e.g., U.S. Patent No. 5,723,127, which is hereby incorporated by reference), 13, 14, 15, 16, 17 and 18 (and its mutant forms), the interferons- α , β and γ , granulocyte-macrophage colony stimulating factor (GM-CSF; see, e.g., U.S. Patent No. 5,078,996, which is hereby incorporated by reference, and ATCC Accession Number 39900), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), and the tumor necrosis factors α and β . Still other adjuvants useful in this invention include a chemokine, including without limitation, MCP-1, MIP-1 α , MIP-1 β , and RANTES. Adhesion molecules, such as a selectin, e.g., L-selectin, P-selectin and E-selectin may also be useful as adjuvants. Still other useful adjuvants include, without limitation, a mucin-like molecule, e.g., CD34, GlyCAM-1 and MadCAM-1, a member of the integrin family such as LFA-1, VLA-1, Mac-1 and p150.95, a member of the immunoglobulin superfamily such as PECAM, ICAMs, e.g., ICAM-1, ICAM-2 and ICAM-3, CD2 and LFA-3, co-stimulatory molecules such as CD40

and CD40L, growth factors including vascular growth factor, nerve growth factor, fibroblast growth factor, epidermal growth factor, B7.2, PDGF, BL-1, and vascular endothelial growth factor, receptor molecules including Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, and DR6. Still another adjuvant molecule includes Caspase (ICE). See, also International Patent Publication Nos. WO98/17799 and WO99/43839, incorporated herein by reference.

Suitable adjuvants used to enhance an immune response further include, without limitation, MPL™ (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton, MT), which is described in U.S. Patent No. 4,912,094, which is hereby incorporated by reference. Also suitable for use as adjuvants are synthetic lipid A analogs or aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa (Hamilton, MT), and which are described in United States Patent No. 6,113,918, which is hereby incorporated by reference. One such AGP is 2-[(R)-3-Tetradecanoyloxytetradecanoylamino] ethyl 2-Deoxy-4-O-phosphono-3-O-[(R)-3-tetradecanoyoxytetradecanoyl]-2-[(R)-3-tetradecanoyloxytetradecanoyl-amino]-β-D-glucopyranoside, which is also known as 529 (formerly known as RC529). This 529 adjuvant is formulated as an aqueous form or as a stable emulsion.

Still other adjuvants include mineral oil and water emulsions, aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, etc., Amphigen, Avridine, L121/squalene, D-lactide-poly(lactide)/glycoside, pluronic polyols, muramyl dipeptide, killed *Bordetella*, saponins, such as Stimulon™ QS-21 (Antigenics, Framingham, MA.), described in U.S. Patent No. 5,057,540, which is hereby incorporated by reference, and particles generated therefrom such as ISCOMS (immunostimulating complexes), *Mycobacterium tuberculosis*, bacterial lipopolysaccharides, synthetic polynucleotides such as oligonucleotides containing a CpG motif (U.S. Patent No.

6,207,646, which is hereby incorporated by reference), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63, LT-R72, PT-K9/G129; see, e.g., International Patent Publication Nos. WO 93/13302 and WO
5 92/19265, incorporated herein by reference.

Also useful as adjuvants are cholera toxins and mutants thereof, including those described in published International Patent Application number WO 00/18434 (wherein the glutamic acid at amino acid position 29 is replaced by another amino
10 acid (other than aspartic acid), preferably a histidine). Similar CT toxins or mutants are described in published International Patent Application number WO 02/098368 (wherein the isoleucine at amino acid position 16 is replaced by another amino acid, either alone or in combination with the
15 replacement of the serine at amino acid position 68 by another amino acid; and/or wherein the valine at amino acid position 72 is replaced by another amino acid). Other CT toxins are described in published International Patent Application number WO 02/098369 (wherein the arginine at amino acid position 25
20 is replaced by another amino acid; and/or an amino acid is inserted at amino acid position 49; and/or two amino acids are inserted at amino acid positions 35 and 36).

The haemolytic activity of the mutant PLY protein may be determined in any suitable manner. One particular protocol as
25 used in the present invention is as follows. Toxin was prepared in serial dilutions in 1.5ml 1 x PBS (Oxoid). An equal volume of 2% (vol/vol) SRBC (sheep red blood cell) was added to each dilution and incubated at 37°C for 30 minutes. Solutions were then centrifuged at 3000rpm for 5 minutes to
30 pellet SRBC membranes or whole cells. Haemoglobin content of the supernatant was read at OD540nm and plotted against toxin concentration to give the degree of haemolysis in relation to toxin concentration. An OD540nm of 0.5 = 50% lysis.

Preferably the mutant protein is non-haemolytic at
35 concentrations of more than 1 µg/ml; more preferably at concentrations of more than 5 µg/ml; still more preferably at

more than 10 µg/ml, at more than 25 µg/ml, or at more than 35 µg/ml; and most preferably at more than 50 µg/ml.

Determination of haemolysis may be carried out as described above.

5 Determination of pore-forming activity may be determined in any suitable manner; a preferred protocol relies on visual inspection of SRBC membranes by means of electron microscopy; this allows the number of pores to be visualised. This protocol is described in more detail below.

10 Several methods may be employed to analyse oligomerising activity of pore-forming toxins. For example, analytical ultracentrifugation, as described by Morgan et al (1993), can be used to study oligomerisation of toxins in solution. A sucrose density gradient can be applied to toxin bound
15 erythrocytes in which oligomers are observed in the high molecular weight fractions and separated from other erythrocyte membrane proteins (Bhakdi et al, 1985; Saunders et al, 1989).

 One particular method of comparing the oligomerisation
20 activity of mutant PLY in solution with that of wild type PLY is to use a fluorescence assay conducted in a similar manner to that described by Search (2002). Briefly, ANS (8-anilino-1-naphthalene-sulphonic acid) (Kodak Ltd.) binds as an extrinsic fluor to PLY. In aqueous solution, ANS has weak fluorescence
25 at 490nm (read with JASCO FP-750 spectrofluorometer) but in a hydrophobic environment ANS fluorescence increases. This phenomenon allows the movement of ANS bound PLY monomers to be tracked in solution. Sodium deoxycholate (BDH Laboratory supplies) can be used to induce oligomerisation of
30 pneumolysin. An increase in fluorescence is observed when wild type PLY plus ANS is treated with sodium deoxycholate as the toxin self-associates bringing the ANS from a hydrophilic to hydrophobic environment. Derivatised PLY, that is PLY chemically modified with dithio(bis)nitrobenzoate to remain
35 monomeric, does not result in an increase in fluorescence at 490nm when treated with sodium deoxycholate. From this

experiment, it is predicted that mutant PLY would give the same result as derivatised PLY if it remains monomeric. If mutant PLY is found to fluoresce to the same extent as WT PLY then it can be concluded that mutant PLY does oligomerise.

5 The toxicity of the proteins and compositions of the invention may be determined directly, by administering the mutant to a non-human test mammal, e.g. a rodent. The toxicity of the mutant may be compared with that of the wild type protein. Suitable indicators of toxicity include
10 survival, animal behaviour, and inflammation (which may be determined by measuring inflammatory cytokine production, e.g. in bronchoalveolar lavage). Suitable protocols are described below in the Examples.

 The present invention further provides a method of
15 preparation of an immunogenic composition, the method comprising the steps of:

 providing an isolated mutant PLY protein with the mutations described herein and having reduced haemolytic activity compared with wild type PLY protein, the mutant
20 protein being antigenic in mammals; and

 conjugating the mutant protein to a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein.

 According to a further aspect of the present invention,
25 there is provided an isolated and purified nucleic acid sequence comprising an isolated nucleic acid sequence encoding a mutant pneumolysin (PLY) protein wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of
30 the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein, or which is complementary to such a nucleic acid sequence, the mutant protein being immunogenic in mammals. Further aspects of the invention provide nucleic acid sequences which are
35 complementary to such sequences.

Nucleic acid sequences can be derived from protein sequences based on the degeneracy of the genetic code.

Nucleic acid sequences of the present invention may comprise additional regulatory sequences, for example,
5 promoters or repressors. The nucleic acid sequences may be comprised in an expression vector, for example, plasmids, artificial chromosomes, expression cassettes and the like.

According to a still further aspect of the present invention, there is provided a recombinant host cell
10 transformed, transfected or infected with a recombinant expression vector comprising an isolated and purified nucleic acid sequence expressing a mutant PLY protein as described herein. In one embodiment, the cell is a prokaryotic cell.

According to a still further aspect of the present invention, there is provided a method of screening candidate
15 mutant PLY proteins for suitability for use in immunogenic compositions, the method comprising the steps of:

providing a mutant PLY protein;
testing the mutant protein for haemolytic activity;
20 testing the mutant protein for oligomerisation activity;
and

comparing the mutant protein haemolytic and oligomerisation activity with those of a non-mutant protein.

Those mutant proteins which have reduced haemolytic and
25 oligomerisation activity compared with the non-mutant form of the protein will be likely to be good candidates for the preparation of immunogenic compositions.

The method may further comprise the step of testing the mutant protein for immunogenic activity in a target mammal.
30 This may comprise the step of contacting the mutant protein with an antibody to the non-mutant protein. This may be performed *in vivo* or *in vitro*.

The invention described herein relates to deletion mutants of pneumolysin which exhibit reduced toxicity,
35 haemolysis and pore formation. The data demonstrate that at

7µg/dose, Δ6 PLY is not detrimental to mice compared to 2µg/dose of WT PLY.

Nevertheless, various mutations created by the present inventors surrounding the N₁₄₃ residue and described herein are still recognised by Western blotting with mAb PLY 4, indicating that this highly antigenic site on PLY has not been altered. The site of mutation has been shown to be highly antigenic by epitope scanning and is recognised by both human sera and rabbit hyper-immune sera (Salo et al, 1993).

All mutants created were confirmed to be forms of pneumolysin. The fact that mAb PLY4 recognises the mutants indicates that the epitope has not been altered to such an extent that it is no longer specific for this antibody. Larger deletions within this area should create mutants that are not recognised by mAbPLY4. As this region has been identified as being highly immunogenic (Salo et al, 1993) it is useful that the site remains intact in the deletions that we have created in terms of use in immunogenic compositions.

The non-toxic mutants described herein are within the site proposed to be involved in oligomerisation (de los Toyos et al, 1996). Further *in vitro* characterisation of one purified toxoid, Δ6 PLY, revealed that it was not cytotoxic to murine fibroblasts or erythrocytes. This suggests that host cell membranes are not lysed by Δ6 PLY because oligomerisation (pore formation) has been prevented. Pores on SRBC membranes treated with WT PLY are readily visible, but pores have not been observed on membranes treated with Δ6 PLY. There were difficulties in fixing Δ6 PLY treated membranes to the grids for visualisation with the EM. This may be due to the agglutination of membranes that are seen in haemolytic assays with Δ6 PLY. The haemagglutination effect observed in haemolytic assays of Δ6 PLY with SRBC suggests that Δ6 PLY monomers still bind to host cell membranes. A labelled form of Δ6 PLY was created which allows visualisation of binding to host cell membranes. From binding assays (data not shown) it was confirmed that Δ6 PLY did bind to the host cell membrane.

There may be a weak affinity between the $\Delta 6$ PLY monomers, allowing cross-linking of monomers but not formation of true oligomers. This cross-linking of $\Delta 6$ PLY monomers in addition to monomers binding to erythrocytes could create the matrix
5 observed in 96-well plates. It is proposed that by creating the $\Delta 6$ PLY mutant, the oligomerisation stage was blocked, but host cell binding/recognition was not abolished.

This hypothesis is supported by the finding that $\Delta 146$ PLY is still capable of associating with cell membranes as
10 determined using eGFP-tagged versions of the mutant and wild type proteins, but that $\Delta 146$ PLY does not form pores in cell membranes. Instead, long chains of protein are seen at the cell surface, which may be self-associated protein which is unable to oligomerise correctly to form pores.

15 *In vivo* treatment with $\Delta 6$ PLY did not result in an increase of the inflammatory cytokine IL-6 24 hours post-treatment. Mice treated with WT PLY were found to produce 10 times more IL-6 than the saline control and $\Delta 6$ PLY treatment. The data established that treatment with $\Delta 6$ PLY did not induce
20 the inflammatory side effects that are associated with native PLY. WT PLY treatment resulted in a localised inflammatory response at the site of administration. This localised IL-6 production in the bronchoalveolar lavage is likely to be from recruited neutrophils (Kadioglu, 2000) and alveolar
25 macrophages that produce more IL-6 than the epithelial cells of the lung tissue (Kerr, personal communication 2003).

Wild type PLY severely damaged lung integrity, but lungs treated with $\Delta 6$ PLY remained healthy. The large amount of total protein observed in the airways of WT PLY treated mice
30 has been characterised as an influx of host proteins (Rubins & Janoff, 1998). PLY has previously been implicated in the disruption of tight junctions (Rayner et al, 1995), allowing host proteins to 'leak' into the airways via the disruption of the capillary/airway barrier. A low inflammatory response and
35 no disruption to the lungs by $\Delta 6$ PLY correlate with $\Delta 6$ PLYs

inability to create pore-forming oligomers in host cell membranes.

Treatment of mice with WT PLY has also been demonstrated to cause a sustained hypothermic response which is not seen in
5 animals treated with $\Delta 146$ PLY.

These and other aspects of the present invention will now be described by way of the following non-limiting examples, and with reference to the accompanying Figures.

10 Examples

Example 1

Site-directed Mutagenesis of Pneumolysin

Eight double amino acid deletions from wild type
15 pneumolysin were created using the Quikchange® site directed mutagenesis kit (Stratagene). The template plasmid was the high expression vector pKK233-3 (Clontech Laboratories) in which PLY was previously inserted. Primers designed to delete the relevant amino acids (see Table 1 below) were ordered from
20 Sigma-Genosys. The following deletions were created to span the N₁₄₃ region of PLY:

W₁₃₄H₁₃₅Q₁₃₆D₁₃₇Y₁₃₈G₁₃₉Q₁₄₀V₁₄₁N₁₄₂N₁₄₃V₁₄₄P₁₄₅A₁₄₆R₁₄₇M₁₄₈Q₁₄₉Y₁₅₀E₁₅₁; where

($\Delta 1$) W₁₃₄H₁₃₅, ($\Delta 2$) Q₁₃₆D₁₃₇, ($\Delta 3$) Y₁₃₈G₁₃₉, ($\Delta 4$) Q₁₄₀V₁₄₁, ($\Delta 5$) V₁₄₄P₁₄₅, ($\Delta 6$) A₁₄₆R₁₄₇, ($\Delta 7$) M₁₄₈Q₁₄₉, ($\Delta 8$) Y₁₅₀E₁₅₁ (see Table 2).

25 N₁₄₂N₁₄₃ was the deletion previously created (Search (2000)), where proteinase K cuts PLY into two fragments.

Table 1 : Primers used to create double amino acid deletions within the PLY gene

Primers	Nucleotide sequences of primers for site-directed mutagenesis
Δ1 fwd	5'-CGATTTGTTGGCTAAGCAAGATTATGGTCAGG-3'
Δ1 rev	5'-CCTGACCATAATCTTGCTTAGCCAACAAATCG-3'
Δ2 fwd	5'-GTTGGCTAAGTGGCATTATGGTCAGGTCAATAATGTCCC-3'
Δ2 rev	5'-GGGACATTATTGACCTGACCATAATGCCACTTAGCCAAC-3'
Δ3 fwd	5'-GGCTAAGTGGCATCAAGATCAGGTCAATAATGTCCC-3'
Δ3 rev	5'-GGGACATTATTGACCTGATCTTGATGCCACTTAGCC-3'
Δ4 fwd	5'-GGCATCAAGATTATGGTAATAATGTCCCAGCTAG-3'
Δ4 rev	5'-CTAGCTGGGACATTATTACCATAATCTTGATGCC-3'
Δ5 fwd	5'-GGTCAGGTCAATAATGCTAGAATGCAGTATG-3'
Δ5 rev	5'-CATACTGCATTCTAGCATTATTGACCTGACC-3'
Δ6 fwd	5'-GGTCAATAATGTCCCAATGCAGTATGAAAAATAACGGCTC-3'
Δ6 rev	5'-GAGCCGTTATTTTTTCATACTGCATTGGGACATTATTGACC-3'
Δ7 fwd	5'-GGTCAATAATGTCCCAGCTAGATATGAAAAATAACGGCTC-3'
Δ7 rev	5'-GAGCCGTTATTTTTTCATATCTAGCTGGGACATTATTGACC-3'
Δ8 fwd	5'-GTCCCAGCTAGAATGCAGAAAATAACGGCTCACAGC-3'
Δ8 rev	5'-GCTGTGAGCCGTTATTTTCTGCATTCTAGCTGGGAC-3'

Note - Reverse primers [rev] are the exact complement and reverse of the forward [fwd] primers with the bases to be
5 deleted removed from the primer.

Table 2 : Bases deleted for each mutation within the PLY gene and amino acids deleted.

Deletion	Bases deleted within the PLY gene	Amino acids deleted
Δ1	TGGCAT (bp400-405)	W ₁₃₄ H ₁₃₅
Δ2	CAAGAT (bp406-411)	Q ₁₃₆ D ₁₃₇
Δ3	TATGGT (bp412-417)	Y ₁₃₈ G ₁₃₉
Δ4	GAGGTC (bp418-423)	Q ₁₄₀ V ₁₄₁
Δ5	GTCCCA (bp430-435)	V ₁₄₄ P ₁₄₅
Δ6	GCTAGA (bp436-441)	A ₁₄₆ R ₁₄₇
Δ7	ATGCAG (bp442-447)	M ₁₄₈ Q ₁₄₉

$\Delta 8$	TATGAA (bp448-453)	Y ₁₅₀ E ₁₅₁
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Example 2

Protein expression and purification

5 Wild type (WT) and mutant PLY was expressed in *Escherichia coli* and harvested as described previously (Mitchell et al, 1989). Cells were disrupted using the benchtop cell disrupter (Constant Systems Ltd) and cytoplasmic proteins obtained by centrifugation at 13,000 rpm for 30
10 minutes. Hydrophobic Interaction Chromatography with a phenyl ether matrix (PE20, Applied Biosystems) was used to purify PLY with the BioCAD (RTM) 700E Perfusion Chromatography Workstation (Applied Biosystems). Eluted fractions were run on SDS-PAGE and coomassie stained using standard protocol and
15 fractions containing pure PLY were pooled.

Example 3

Quantitative Haemolytic Assay

Haemolytic activity of purified protein was assessed
20 using an assay based on that reported by Walker et al., (1987) using a 2% (vol/vol) sheep red blood cell (SRBC) (E & O laboratories) solution in 1 x Phosphate Buffered Saline (PBS) (Oxoid). Pooled fractions were concentrated using minicon B15 clinical sample concentrators (Millipore). Toxin was prepared
25 in serial dilutions in 1.5ml 1 x PBS (Oxoid). An equal volume of 2% (vol/vol) SRBC was added to each dilution and incubated at 37°C for 30 minutes. Solutions were then centrifuged at 3000rpm for 5 minutes to pellet SRBC membranes or whole cells. Haemoglobin content of the supernatant was read at OD540nm and
30 plotted against toxin concentration to give the degree of haemolysis in relation to toxin concentration. An OD540nm of 0.5 = 50% lysis.

Crude haemolytic analysis revealed that four of these mutants, deletions 5-8, were non-haemolytic. Further analysis
35 of purified $\Delta 6$ PLY (A₁₄₆R₁₄₇) in a quantitative haemolytic assay

(Fig. 3) revealed that it was non-lytic at concentrations of 50µg/ml whereas <1µg/ml of WT PLY was haemolytic to SRBC. Purified preparations of Δ5 PLY and Δ6 PLY were observed to agglutinate erythrocytes in 96-well micro-titre plates but not lyse the cells; this effect was concentration dependent.

Example 4

Electron Microscopy

200µl 2% (v/v) SRBC solution was incubated with an equal volume of 0.2mg/ml WT PLY or Δ6 PLY at 37°C for 20 minutes then centrifuged with benchtop centrifuge to pellet the SRBC membranes. Membranes were washed with dH₂O x 3 and resuspended in 100µl dH₂O. 5µl of suspension was fixed onto carbon-coated grids and negatively stained with 1% phosphotungstate acid, pH6.8. Magnification was at x 25000 using an LEO 912 Energy Filter Transmission Electron Microscope.

30-40µm pores were visualised on erythrocyte membranes treated with 0.2mg/ml WT PLY (Fig. 5). In contrast, pores were not visualised on membranes treated with 0.2mg/ml Δ6 PLY.

Example 5

Western Blotting

PLY mutants created by site-directed mutagenesis were detected in Western blots using standard techniques. Blots were incubated with polyclonal anti-PLY serum from rabbit or monoclonal PLY 4 anti-PLY serum from mouse (de los Toyos et al, 1996) and then incubated with the relevant HRP-linked antibody (Amersham Life Sciences) and developed.

Of the eight double amino acid deletions created, all were recognised by Western blotting with polyclonal anti-PLY serum (not shown) and by mAb PLY 4 (Fig. 2) prepared by de los Toyos et al (1996).

Example 6L929 Killing Assay

L929 murine fibroblasts (ECACC, no.85011425) were cultured in RPMI 1640 media + 10% Foetal Bovine Serum (FBS) (Gibco), passaged and transferred to a 96-well plate and incubated for 24h at 37°C, 5% CO₂. Serial dilutions of purified WT PLY and mutant Δ6 PLY toxin were prepared in RPMI 1640 media from a stock concentration of 0.05mg/ml and added to the L929 fibroblasts. Cell viability upon 24h incubation with PLY was assessed using MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) which is degraded by mitochondrial activity into a purple formazan precipitate. MTT in wells with dead cells will remain yellow. Optical density was read at 540nm with an MRX plate reader (Dynatech Laboratories).

Cytotoxicity assays with L929 murine fibroblasts were run to assess the toxicity of mutant Δ6 PLY compared to WT PLY (Fig. 4). At concentrations of 30μg/ml, Δ6 PLY was not toxic to fibroblasts, whereas < 500 pg/ml of WT PLY was cytotoxic.

20

Example 7In vivo Cytokine Analysis

Eight week-old MF-1 mice (Harlan) were lightly anaesthetised with 2% halothane/1.5% oxygen (1.5 litre/min) (Zeneca). Purified LPS-free WT PLY was administered intranasally at 2μg/dose and Δ6 PLY at 7μg/dose (9.928ng LPS/dose) in 50μl volumes with a saline group as a control (n=4 for each treatment). Lipopolysaccharide (LPS) content of purified toxin was determined using the Limulus Amebocyte Lysate (LAL) Kinetic-QCL Kit (BioWhittaker) and run according to manufacturer's instructions. Mice were monitored to a 24h end-point. Serum, bronchoalveolar lavage and lung tissue samples were recovered and processed as described previously (Kerr, et al. 2002). Cytokine levels were measured with commercial cytokine ELISA kits for Interleukin (IL)-6, Interferon (IFN)-γ (Pharmigen) and Tumor Necrosis Factor

35

(TNF)- α (R&D systems, UK). Total protein levels in the lavage were measured using standard Bradford Assay.

Non-parametric analysis by Mann-Whitney U test was used to measure cytokine and total protein levels where $p < 0.05$ was considered statistically significant. Values are expressed as medians \pm 1 median absolute deviation (MAD) using Statview (Abacus Concepts).

As part of *in vivo* toxicity studies, gross symptoms were ascertained. All mice survived 24 hours post-treatment except one from the WT PLY group. Mice treated with $\Delta 6$ PLY and saline recovered from the anaesthetic quicker than mice given WT PLY. Behaviour of $\Delta 6$ PLY treated mice was similar to that of the saline control but WT PLY treated mice exhibited piloerection, laboured breathing and a hunched stance over a 6- hour period, recovering within the 24-hour time scale.

Next, an inflammatory cytokine analysis was performed. IL-6 production was measured as a marker of toxicity of PLY to the host. There was a greater than 10-fold increase in IL-6 levels in the bronchoalveolar lavage of WT PLY treated mice (Fig. 7) compared to $\Delta 6$ PLY treatment ($p < 0.05$) and the saline control ($p < 0.05$). Treatment with WT PLY induces inflammation in the host airways whereas treatment with $\Delta 6$ PLY does not. The median IL-6 level in WT treated bronchoalveolar lavage was 416pg/ml (range of 335-2225pg/ml) whereas the background IL-6 level was low (59pg/ml) with no increase in mice treated with $\Delta 6$ PLY (36pg/ml) (see Table 3 below). An increase in IL-6 levels was observed in lung tissue of WT treated mice ($p < 0.05$) compared to the saline control (Fig. 6). There was no significant IL-6 increase in lung tissue of $\Delta 6$ treated mice compared to the saline treatment. Measurements of IFN- γ and TNF- α were not significant between treatments 24h post-administration (data not shown).

Table 3: IL-6 median (min-max) levels in bronchoalveolar lavage 24h post treatment

Treatment (i.n.)	Lung Tissue (pg/ml)	Lung Lavage (pg/ml)	P values (*=significant)		
				Lung	Lavage
NaCl	117 (103-139)	59 (19-113)	NaCl/ Δ 6	0.3865	0.7728
Δ 6	147 (73-209)	36 (30-132)	Δ 6/WT	0.1573	0.0339*
Wild type (WT)	171 (168-448)	416 (335-2225)	NaCl/WT	0.0339*	0.0339*

Total protein levels (Fig. 8) were measured in the bronchoalveolar lavage to assess lung integrity. Increases in protein levels were not observed for Δ 6 PLY treated mice compared to healthy lavage samples. Airways of WT PLY treated mice had large amounts of protein (3.57mg/ml) in them compared to a background total protein level of 0.23mg/ml for the saline control group (Fig. 8).

10 Example 8

Mouse Immunogenicity Studies

A mouse immunogenicity study was performed to compare responses of the wild type PLY protein to the Δ 5, 6 and 7 mutant proteins. All immunogenic compositions were prepared at 5 μ g rPLY/dose in the presence of a combination of adjuvants, AlPO₄ (0.2 mg) and MPL-SE (50 μ g). AlPO₄ (0.2 mg) and MPL-SE (50 μ g) in phosphate-buffered saline (PBS) was used as a negative control.

Groups of 5 female, CD-1 mice, age 6-8 weeks, were immunized intraperitoneally and received 2 booster doses at 2 week intervals. Blood was collected retro-orbitally at weeks 0, 2, 4, and 6. Individual serum was assayed and GMTs represented an end point at 0.3. Week 0 antibodies were all <50. As shown in Table 4, the three PLY mutants elicited antibodies in the mice which were comparable to those elicited by wild type PLY.

Table 4: Serum IgG Antibody Responses to Mutant Recombinant Pneumolysin (rPLY) in Mice

Immunogen	rPL Dose (μ g)	GMT Antibodies for rPL Week		
		2	4	6
PBS	-	50	50	50
rPL (nlrPL 01-01)	5.0	16,456	806,776	3,104,055
rPL Wild type (PLY C73)	5.0	11,420	578,645	997,727
rPL Δ 5	5.0	3,892	424,253	1,375,535
rPL Δ 6	5.0	18,679	999,574	1,452,972
rPL Δ 7	5.0	15,920	2,266,935	1,246,988

5

Example 9

Generation of anti-PLY Antibodies

Levels of anti-PLY antibodies raised in mice immunized with wild type PLY or Δ 6 PLY were determined by immunizing MF-1 mice with an initial subcutaneous injection with 20 μ g WT PLY or Δ 6 PLY, each with 100 μ g Alum/ 100 μ l dose. Mice were then boosted twice with the same dosage. Serum was collected on day 47 of the immunization protocol and analysed for anti-PLY IgG antibody. Antibody dilution curves are displayed in Figure 9 as the group mean OD_{490nm} \pm SEM against the serum serial dilution. An initial dilution of serum to 1/1000 was used as more concentrated samples resulted in complete saturation of the substrate. Figure 9 demonstrates that high levels of antibodies were produced in response to both Δ 6 PLY + Alum and WT PLY+ Alum, but not to the Alum only control group.

Next, the ability of anti-PLY antibodies to neutralise the haemolytic activity was assessed. Anti-PLY antibodies in the Δ 6 PLY and wild type PLY treated groups were observed to

completely neutralise 2.5 Haemolytic Units (HU) of PLY to a titre of 1000-2400 in a haemolytic assay (where neutralising ability is expressed as the reciprocal of the antibody dilution that completely neutralises 2.5HU of PLY) (data not shown). This demonstrates that a neutralising site on PLY is recognised and bound to by the antibodies produced in response to immunisation with $\Delta 6$ PLY + Alum and WT PLY + Alum. Because $\Delta 6$ PLY is non-toxic and does not induce the *in vivo* levels of cytokine production observed with wild type PLY treatment, $\Delta 6$ PLY is therefore a more favourable protein than wild type PLY for use as an immunogenic carrier protein.

Example 10

Effect of a Single Amino Acid Deletion

A mutant PLY was generated which had a single amino acid deletion: the alanine at amino acid 146 was deleted ($\Delta A146$ PLY). As shown in Figure 10, this single deletion ($\Delta A146$) also resulted in a non-haemolytic form of PLY. $\Delta A146$ PLY was not haemolytic to SRBC to concentrations $>100\mu\text{g/ml}$, whereas wild type PLY was haemolytic at concentrations $<1\mu\text{g/ml}$. Production of this mutant was confirmed by sequencing and by Western blotting of the expressed protein with polyclonal anti-PLY serum (data not shown).

Example 11

Haemolytic activity of PLY mutants in comparison to PLY W433F

The haemolytic activity of the deletion mutants $\Delta 6$, $\Delta 7$, $\Delta 8$ PLY and $\Delta A146$ PLY against human erythrocytes was compared with that of WT PLY and PLY mutant carrying the substitution W433F, which has previously been described to possess only 1% of the haemolytic activity of WT PLY (see WO90/06951).

Figure 11 shows that, as expected, the W433F mutant shows ~1% of the haemolytic activity of wild type PLY. However the

deletion mutants do not cause lysis of human erythrocytes at all.

Example 12

5 Binding of GFP-tagged PLY to erythrocyte membranes

Fluorescence microscopy was used to visualise erythrocytes treated with eGFP-tagged forms of WT PLY and $\Delta 6$ PLY.

10 Erythrocyte ghosts were prepared from human blood by repeated washing with distilled water. Erythrocyte ghosts generated from 0.1ml human blood were incubated with 50 μ g EGFP-PLY or 50 μ g $\Delta 6$ EGFP-PLY in 1ml 1 \times PBS for 30 min at 37°C. The ghost membranes were pelleted, washed \times 3 in PBS and were visualized by fluorescence microscopy using a Zeiss Axioscop 20.

15

The results (not shown) demonstrate that the binding of $\Delta 6$ PLY to membranes is substantially the same as that of WT PLY.

Example 13

20 Analysis of pore formation using Transmission Electron Microscopy.

Electron microscopy was performed as described above (Example 4) for negatively stained horse erythrocyte membranes treated with 0.2mg/ml wild type pneumolysin, 0.2mg/ml W433F PLY, and 0.2mg/ml $\Delta A146$ PLY.

Pores were observed on membranes treated with wt PLY and W433F but not on membranes treated with $\Delta A146$ PLY. Instead, $\Delta A146$ PLY treatment resulted in the formation of long chains, thought to contain self-associated toxin that is unable to oligomerise to form pores. (Data not shown.)

35 Thus $\Delta 6$ PLY retains the membrane-binding properties of wild type PLY but does not form pores in cell membranes.

Example 14Cytotoxicity of pneumolysin mutants to murine L929 fibroblasts

- 5 The cytotoxicity of WT PLY, PLY W433F, and deletion mutants $\Delta 6$, $\Delta 7$, $\Delta 8$ PLY and $\Delta A146$ PLY against murine L929 fibroblasts was determined as described in Example 6. human erythrocytes was compared with that of WT PLY and
- 10 The W433F PLY mutant was found to be cytotoxic at 10 μ g/ml and above, whereas the deletion mutants were non-toxic in this assay (Figure 12).

Example 15

- 15 Cytotoxicity of pneumolysin mutant $\Delta A146$ PLY to RBL-2H3 mast cells

The cytotoxicity of $\Delta A146$ PLY against rat RBL-2H3 mast cells was assessed using a degranulation assay.

- 20 The assay was carried out as described by Stassen et al (2003) using 10⁴ cells/well, incubated with wild type PLY or $\Delta A146$ PLY for 90 minutes.
- 25 Release of β -hexosaminidase from mast cell granules was measured, which gives a direct measure of the degranulation of the cells in response to the toxin. $\Delta A146$ PLY did not cause mast cell degranulation (Figure 13).

30 Example 16

Analysis of murine core body temperature following treatment with wt PLY or $\Delta A146$ PLY

- Balb/c mice were implanted with telemetry chips which enable acquisition of core body temperature (Tc). Mice were treated
- 35 with 1 μ g wt PLY, 1 μ g $\Delta A146$ PLY, or saline solution alone.

As shown in Figure 14, Treatment with WT PLY resulted in a severe hypothermic response with Tc dropping to 28°C. This Tc was sustained for 6 hours after which there was an increase in Tc by ~0.6°C/hour and by 24 hours this was similar to the Tc of the control group, though still statistically significant. Treatment with ΔA146 PLY did not result in hypothermia and the median Tc was comparable to the saline control group.

Thus, treatment of mice with WT PLY resulted in a sustained hypothermic response that was not observed following treatment with the same amount of ΔA146 PLY.

While the invention has been described in conjunction with the exemplary embodiments described above, many equivalent modifications and variations will be apparent to those skilled in the art when given this disclosure. Accordingly, the exemplary embodiments of the invention set forth are considered to be illustrative and not limiting. Various changes to the described embodiments may be made without departing from the spirit and scope of the invention. All references cited herein are expressly incorporated by reference.

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CLAIMS

1. An isolated mutant pneumolysin (PLY) protein,
wherein the mutant PLY protein differs from the wild type PLY
5 protein by the presence of a mutation within the region of
amino acids 144 to 161 of the wild type sequence, such that
the toxicity of the mutant is reduced relative to that of the
wild-type protein

10 2. The protein of claim 1, wherein the mutant PLY
protein differs from the wild type PLY protein by the deletion
or substitution of one or more amino acids within the region
of amino acids 144 to 161 of the wild type sequence.

15 3. The protein of claim 1, wherein the mutant PLY
protein differs from the wild type PLY protein by the presence
of a mutation within the region of amino acids 144 to 151 of
the wild type sequence.

20 4. The protein of claim 3, wherein the mutant PLY
protein differs from the wild type protein by the substitution
or deletion of one or more amino acids within the region of
amino acids 144 to 151 of the wild type sequence.

25 5. The protein of claim 4, wherein alanine 146 is
substituted or deleted.

6. The protein of claim 4, wherein the mutant PLY
protein differs from the wild type protein by the deletion of
30 two adjacent amino acids within the region of amino acids 144
to 151 of the wild type sequence.

7. The protein of claim 6, wherein amino acids valine
144 and proline 145 are deleted.

8. The protein of claim 6, wherein amino acids alanine 146 and arginine 147 are deleted.

9. The protein of claim 6, wherein amino acids methionine 148 and glutamine 149 are deleted.

10. The protein of claim 6, wherein amino acids tyrosine 150 and glutamic acid 151 are deleted.

11. The protein of any one of claims 1 to 10, wherein the protein further comprises at least one amino acid substitution or deletion in at least one of the regions of amino acids 257-297, 367-397 or 424-437 of the wild type sequence.

15

12. An immunogenic conjugate comprising:

(a) a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein; and

(b) an isolated mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein.

25

13. The immunogenic conjugate of claim 12 wherein the mutant PLY protein differs from the wild type PLY protein by the deletion or substitution of one or more amino acids within the region of amino acids 144 to 161 of the wild type sequence.

30

14. The immunogenic conjugate of claim 12 wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 151 of the wild type sequence.

35

15. The immunogenic conjugate of claim 13, wherein the mutant PLY protein differs from the wild type protein by the substitution or deletion of one or more amino acids within the region of amino acids 144 to 151 of the wild type sequence.

16. The immunogenic conjugate of claim 15 wherein alanine 146 is substituted or deleted.

17. The immunogenic conjugate of claim 15, wherein the mutant PLY protein differs from the wild type protein by the deletion of two adjacent amino acids within the region of amino acids 144 to 151 of the wild type sequence.

18. The immunogenic conjugate of claim 17, wherein amino acids valine 144 and proline 145 of the PLY protein are deleted.

19. The immunogenic conjugate of claim 17, wherein amino acids alanine 146 and arginine 147 of the PLY protein are deleted.

20. The immunogenic conjugate of claim 17, wherein amino acids methionine 148 and glutamine 149 of the PLY protein are deleted.

21. The immunogenic conjugate of claim 17, wherein amino acids tyrosine 150 and glutamic acid 151 of the PLY protein are deleted.

22. The immunogenic conjugate of any one of claims 12 to 21, wherein the saccharide, oligosaccharide or polysaccharide is derived from *Streptococcus pneumoniae*.

23. The immunogenic conjugate of any one of claims 12 to 22, wherein the protein further comprises at least one amino

acid substitution or deletion in at least one of the regions of amino acids 257-297, 367-397 or 424-437 of the wild type sequence.

5 24. An isolated and purified nucleic acid sequence comprising a nucleic acid sequence a) encoding a mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the
10 wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein; or b) which is complementary to a nucleic acid sequence defined in a).

 25. The isolated and purified nucleic acid sequence of
15 claim 24, wherein the mutant PLY protein differs from the wild type PLY protein by the deletion or substitution of one or more amino acids within the region of amino acids 144 to 161 of the wild type sequence.

20 26. The isolated and purified nucleic acid sequence of claim 24, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 151 of the wild type sequence.

25 27. The isolated and purified nucleic acid sequence of claim 26, wherein alanine 146 is substituted or deleted.

 28. The isolated and purified nucleic acid sequence of claim 26, wherein the mutant PLY protein differs from the wild
30 type protein by the substitution or deletion of one or more amino acids within the region of amino acids 144 to 151 of the wild type sequence.

 29. The isolated and purified nucleic acid sequence of
35 claim 28, wherein the mutant PLY protein differs from the wild type protein by the deletion of two adjacent amino acids

within the region of amino acids 144 to 151 of the wild type sequence.

30. The isolated and purified nucleic acid sequence of
5 claim 29, wherein amino acids valine 144 and proline 145 are deleted.

31. The isolated and purified nucleic acid sequence of
claim 29, wherein amino acids alanine 146 and arginine 147 are
10 deleted.

32. The isolated and purified nucleic acid sequence of
claim 29, wherein amino acids methionine 148 and glutamine 149
are deleted.

15

33. The isolated and purified nucleic acid sequence of
claim 29, wherein amino acids tyrosine 150 and glutamic acid
151 are deleted.

20 34. The isolated and purified nucleic acid sequence of
any one of claims 24 to 33, wherein the protein further
comprises at least one amino acid substitution or deletion in
at least one of the regions of amino acids 257-297, 367-397 or
424-437 of the wild type sequence.

25

35. A recombinant expression vector comprising an
isolated and purified nucleic acid sequence comprising a
nucleic acid sequence of any one of claims 24 to 34.

30 36. A recombinant host cell transformed, transfected or
infected with a recombinant expression vector of claim 35.

37. A method of producing an isolated mutant pneumolysin
(PLY) protein, wherein the mutant PLY protein differs from the
35 wild type PLY protein by the presence of a mutation within the
region of amino acids 144 to 161 of the wild type sequence,

such that the toxicity of the mutant is reduced relative to that of the wild-type protein, which comprises: a) transforming, transfecting or infecting a host cell with the recombinant expression vector of claim 35 and culturing the host cell under conditions which permit the expression of said mutant PLY protein by the host cell; and b) recovering the mutant PLY protein from the culture.

38. An immunogenic composition which comprises: a) an isolated mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein; and b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

39. The immunogenic composition of claim 38, wherein the mutant PLY protein differs from the wild type PLY protein by the deletion or substitution of one or more amino acids within the region of amino acids 144 to 161 of the wild type sequence.

40. The immunogenic composition of claim 38, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 151 of the wild type sequence.

41. The immunogenic composition of claim 40, wherein the mutant PLY protein differs from the wild type protein by the deletion or substitution of one or more amino acids within the region of amino acids 144 to 151 of the wild type sequence.

42. The immunogenic composition of claim 41, wherein alanine 146 is substituted or deleted.

43. The immunogenic composition of claim 41, wherein the mutant PLY protein differs from the wild type protein by the deletion of two adjacent amino acids within the region of amino acids 144 to 151 of the wild type sequence.

5

44. The immunogenic composition of claim 43, wherein amino acids valine 144 and proline 145 are deleted.

45. The immunogenic composition of claim 43, wherein
10 amino acids alanine 146 and arginine 147 are deleted.

46. The immunogenic composition of claim 43, wherein amino acids methionine 148 and glutamine 149 are deleted.

15 47. The immunogenic composition of claim 43, wherein amino acids tyrosine 150 and glutamic acid 151 are deleted.

48. The immunogenic composition of any one of claims 38 to 47, wherein the protein further comprises at least one
20 amino acid substitution or deletion in at least one of the regions of amino acids 257-297, 367-397 or 424-437 of the wild type sequence.

49. An immunogenic composition which comprises: a) an
25 immunogenic conjugate comprising:
 (i) a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein; and
 (ii) an isolated mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild
30 type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein; and
35 b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

50. The immunogenic composition of claim 49, wherein the mutant PLY protein differs from the wild type PLY protein by the deletion or substitution of one or more amino acids within the region of amino acids 144 to 161 of the wild type sequence.

51. The immunogenic composition of claim 49, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 151 of the wild type sequence.

52. The immunogenic composition of claim 51, wherein the mutant PLY protein differs from the wild type protein by the substitution or deletion of one or more amino acids within the region of amino acids 144 to 151 of the wild type sequence.

53. The immunogenic composition of claim 52, wherein alanine 146 is substituted or deleted.

54. The immunogenic composition of claim 52, wherein the mutant PLY protein differs from the wild type protein by the deletion of two adjacent amino acids within the region of amino acids 144 to 151 of the wild type sequence.

55. The immunogenic composition of claim 54, wherein amino acids valine 144 and proline 145 of the PLY protein are deleted.

56. The immunogenic composition of claim 54, wherein amino acids alanine 146 and arginine 147 of the PLY protein are deleted.

57. The immunogenic composition of claim 54, wherein amino acids methionine 148 and glutamine 149 of the PLY protein are deleted.

58. The immunogenic composition of claim 54, wherein amino acids tyrosine 150 and glutamic acid 151 of the PLY protein are deleted.

5

59. The immunogenic composition of any one of claims 49 to 58, wherein the saccharide, oligosaccharide or polysaccharide is derived from *Streptococcus pneumoniae*.

10

60. The immunogenic composition of claim 59, wherein there are a plurality of *Streptococcus pneumoniae* serotypes.

15

61. The immunogenic composition of any one of claims 49 to 60, wherein the protein further comprises at least one amino acid substitution or deletion in at least one of the regions of amino acids 257-297, 367-397 or 424-437 of the wild type sequence.

20

62. A method of prophylaxis for a mammal, the method comprising the step of administering to a subject mammal an immunogenic composition which comprises: a) an isolated mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild type PLY protein by the deletion of one or more amino acids within the region of amino acids 144 to 161 of the wild type sequence; and b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

25

63. A method of prophylaxis for a mammal, the method comprising the step of administering to a subject mammal an immunogenic composition which comprises: a) an immunogenic conjugate comprising:

30

(i) a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or protein; and

(ii) an isolated mutant pneumolysin (PLY) protein,

35

wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation

within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein; and

- 5 b) one or more of a physiologically acceptable adjuvant, diluent or carrier.

64. Use of a mutant PLY protein or immunogenic conjugate thereof in the preparation of an immunogenic composition for
10 the prophylaxis or treatment of bacterial infection, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type
15 protein.

65. An isolated mutant PLY protein or immunogenic conjugate thereof for use in a method of medical treatment, wherein the mutant PLY protein differs from the wild type PLY
20 protein by the presence of a mutation within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein.

25 66. A method of preparation of an immunogenic composition, the method comprising the steps of:

 providing an isolated mutant PLY protein, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids
30 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild-type protein; and

 conjugating the mutant protein to a saccharide, oligosaccharide, polysaccharide, peptide, polypeptide or
35 protein.

67. The method of claim 66, wherein the mutant protein is conjugated to a *Streptococcus pneumoniae* polysaccharide.

68. A method of screening candidate mutant PLY proteins
5 for suitability for use in immunogenic compositions, the
method comprising the steps of:
 providing a mutant PLY protein;
 testing the mutant protein for haemolytic activity;
 testing the mutant PLY protein for oligomerisation
10 activity; and
 comparing the mutant PLY protein haemolytic and
oligomerisation activity with those of a non-mutant protein.

Pneumolysin from *Streptococcus pneumoniae*
Length: 471 AA g.i. 47403 (X52474)

```
1  MANKAVNDFI LAMNYDKKKL LTHQGESIEN RFIKEGNQLP DEFVVIERKK
51  RSLSTNTSDI SVTATNDSRL YPGALLVVDE TLENNPTLL AVDRAPMTYS
101 IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVPARMQY
151 EKITAHSMEQ LKVKEFGSDFE KTGNSLDIDF NSVHSGEKQI QIVNFKQIYY
201 TVSVDAVKNP GDVFQDTVTV EDLKQRGISA ERPLVYISSV AYGRQVYLKL
251 ETTSKSDEVE AAFEALIKGV KVAPQTEWKQ ILDNTEVKAV ILGGDPSSGA
301 RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATEFQNSTDY
351 VETKVTAYRN GDLLLDHSGA YVAQYYITWN ELSYDHQGKE VLTPKAWDRN
401 GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAWEEWRTVY EKTDLPLVRK
451 RTISIWGTTL YPQVEDKVEN D
```

Figure 1

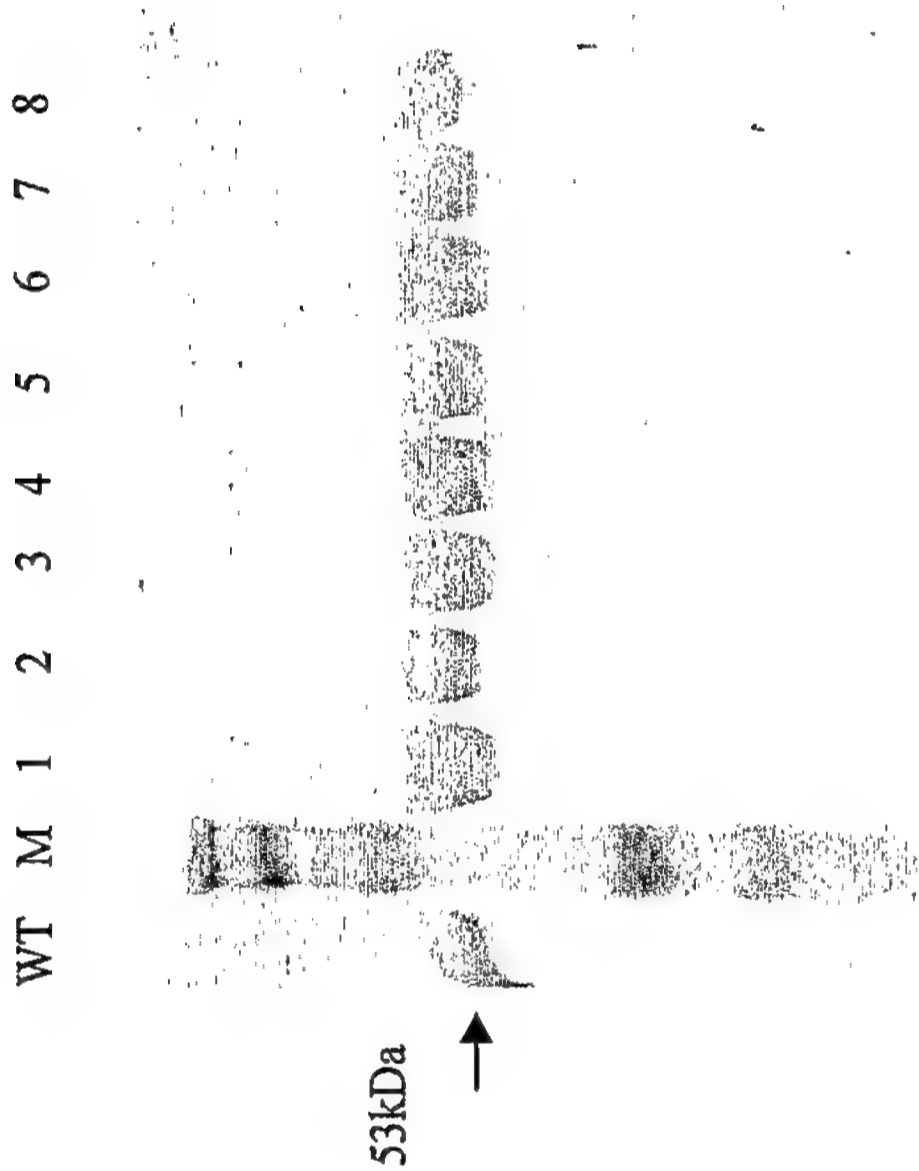


Figure 2

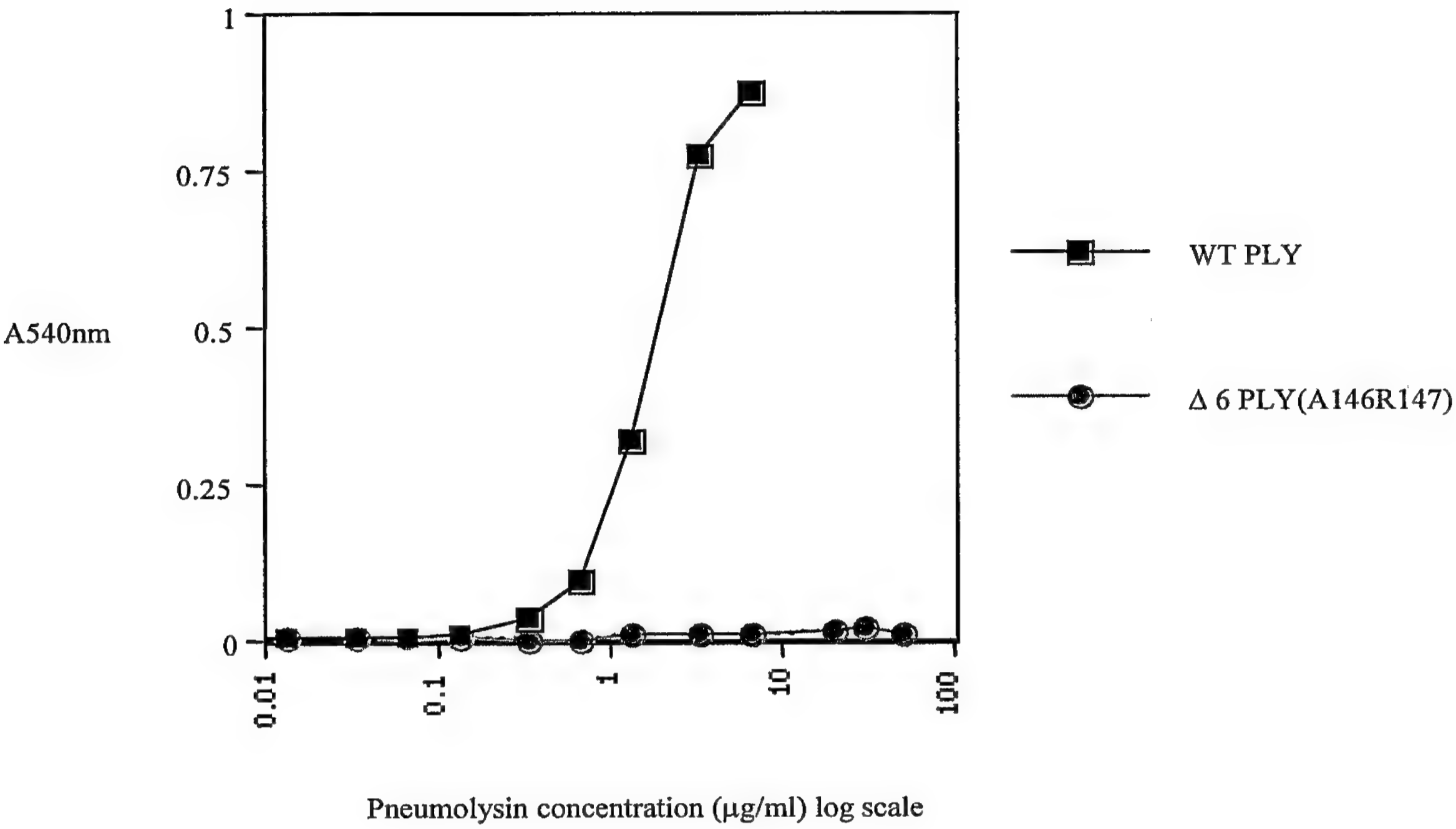


Figure 3

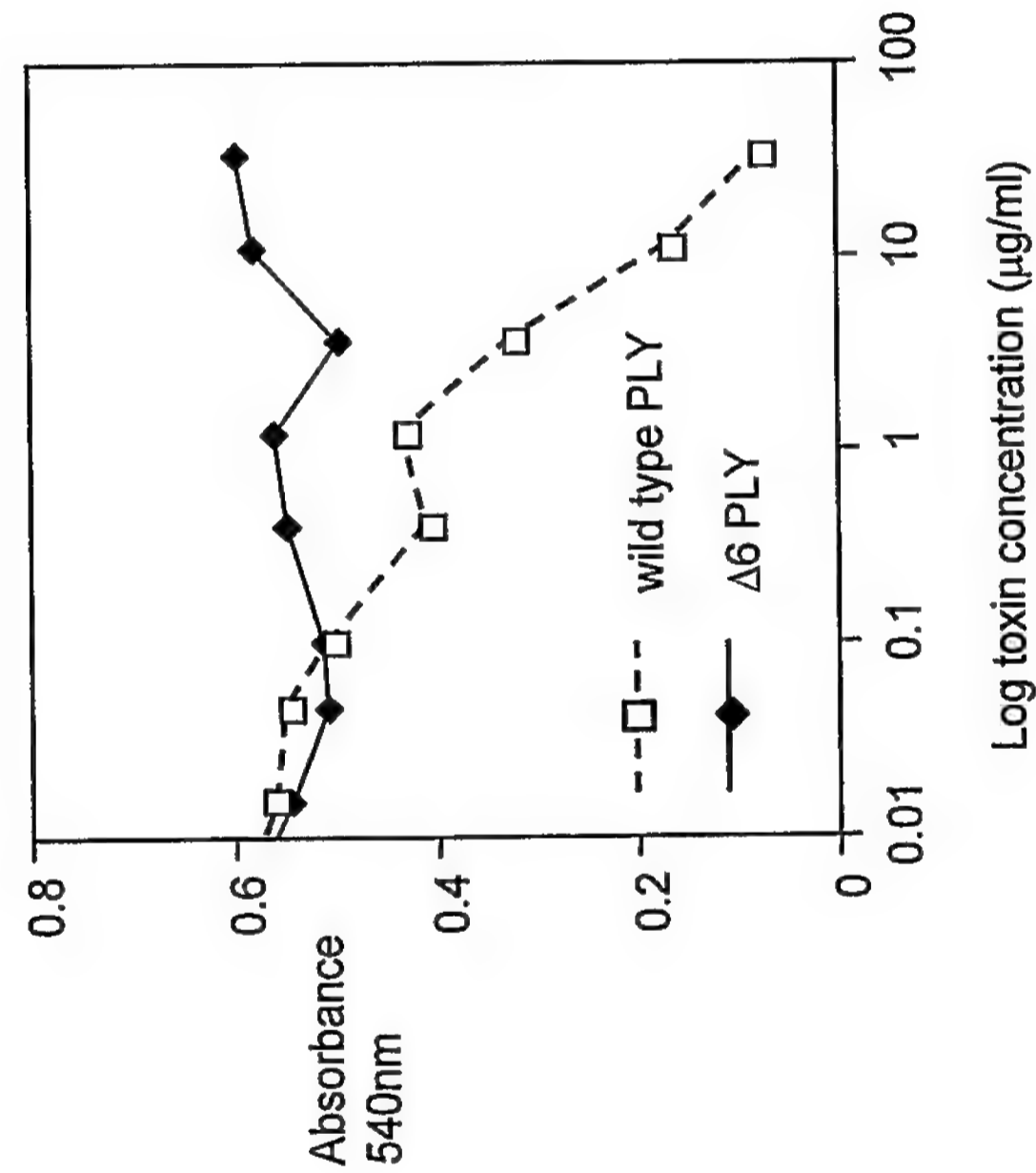


Figure 4

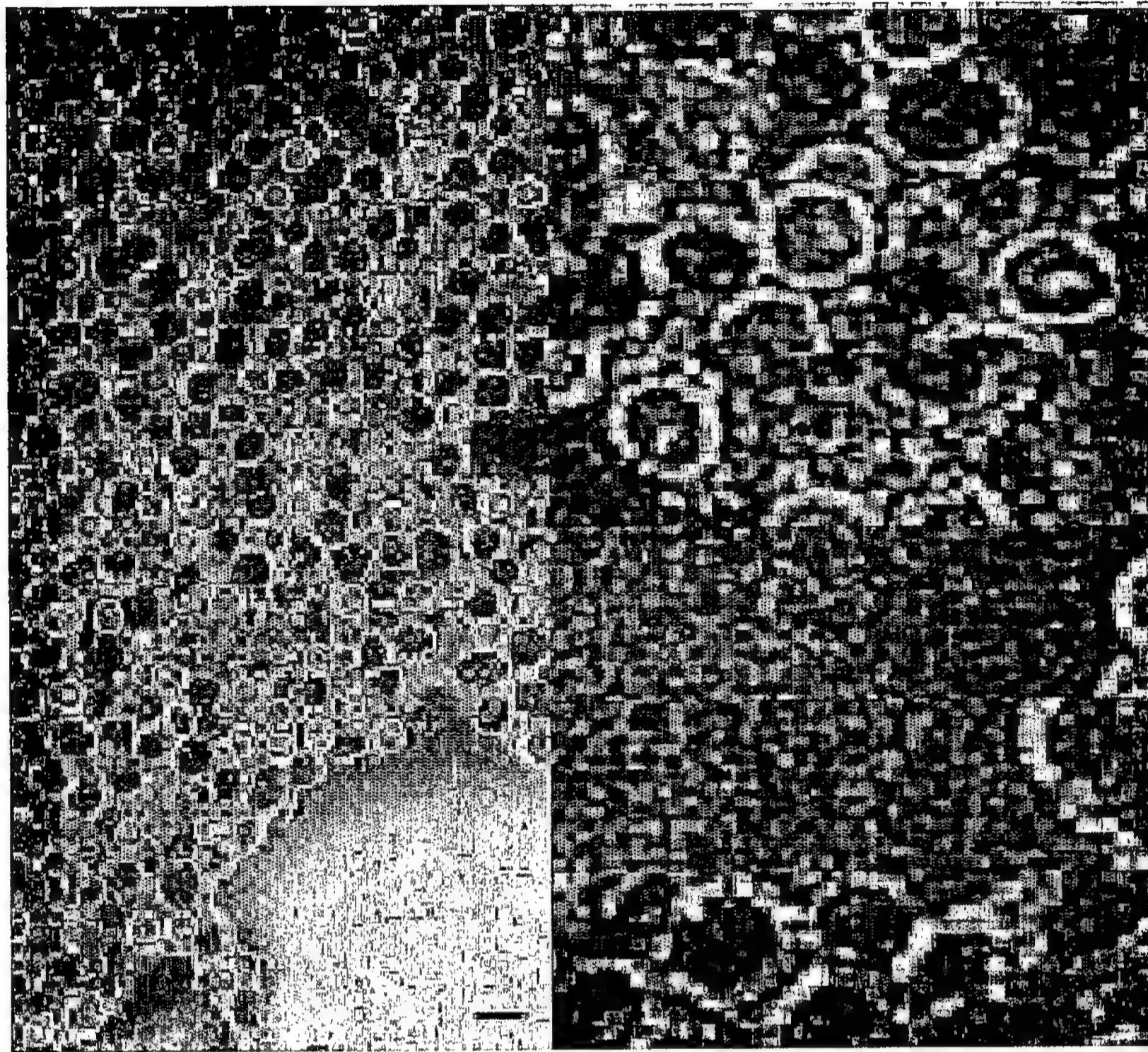


Figure 5

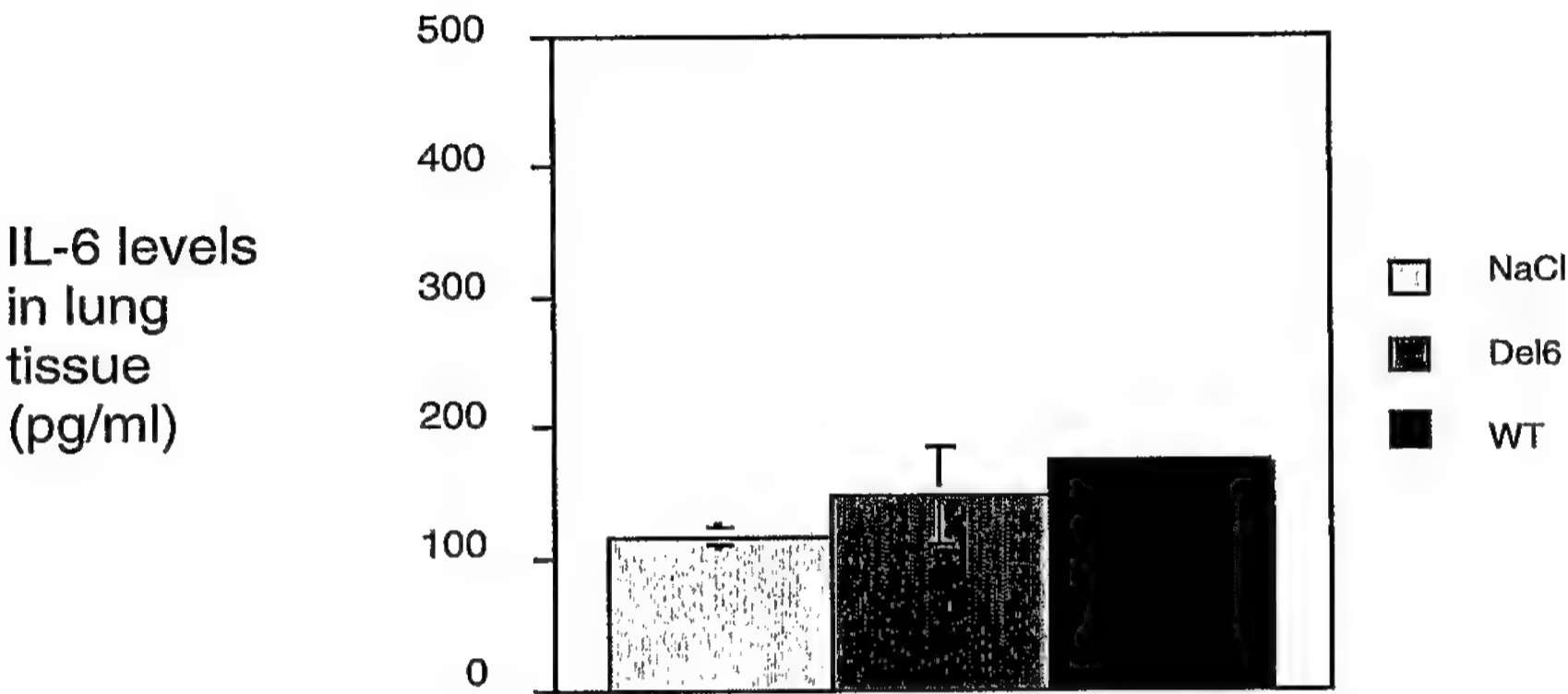


Figure 6

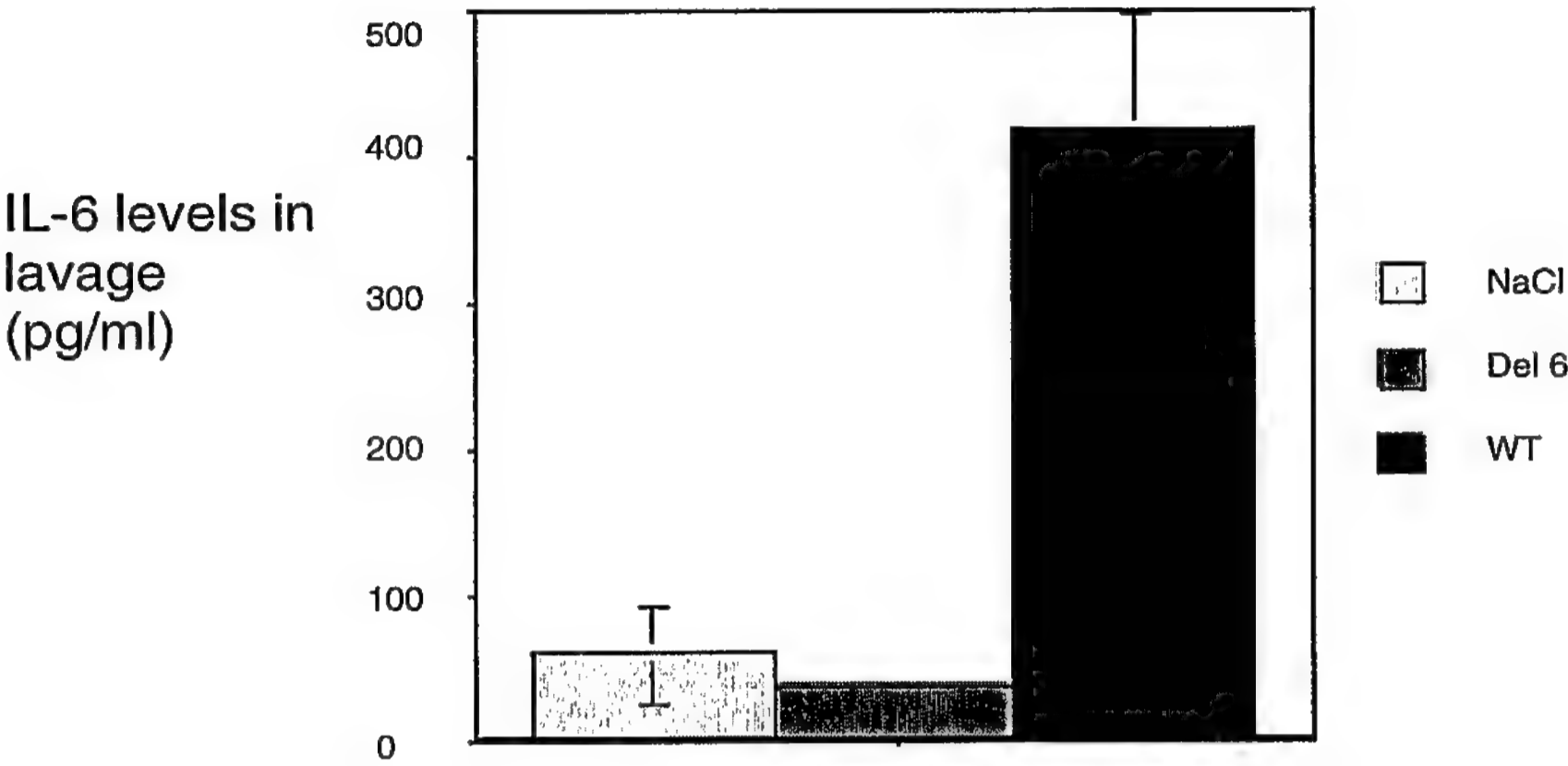


Figure 7

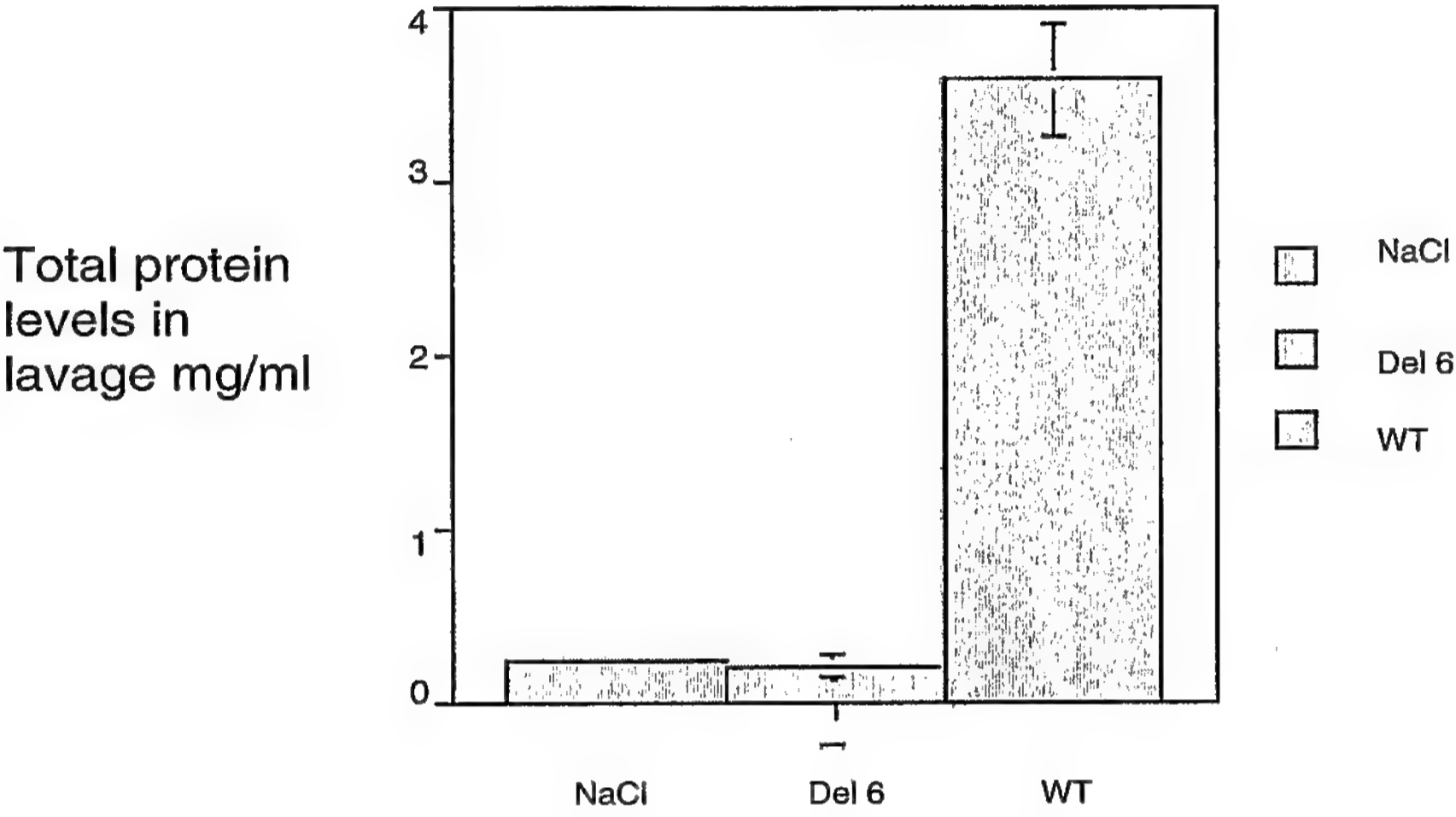


Figure 8

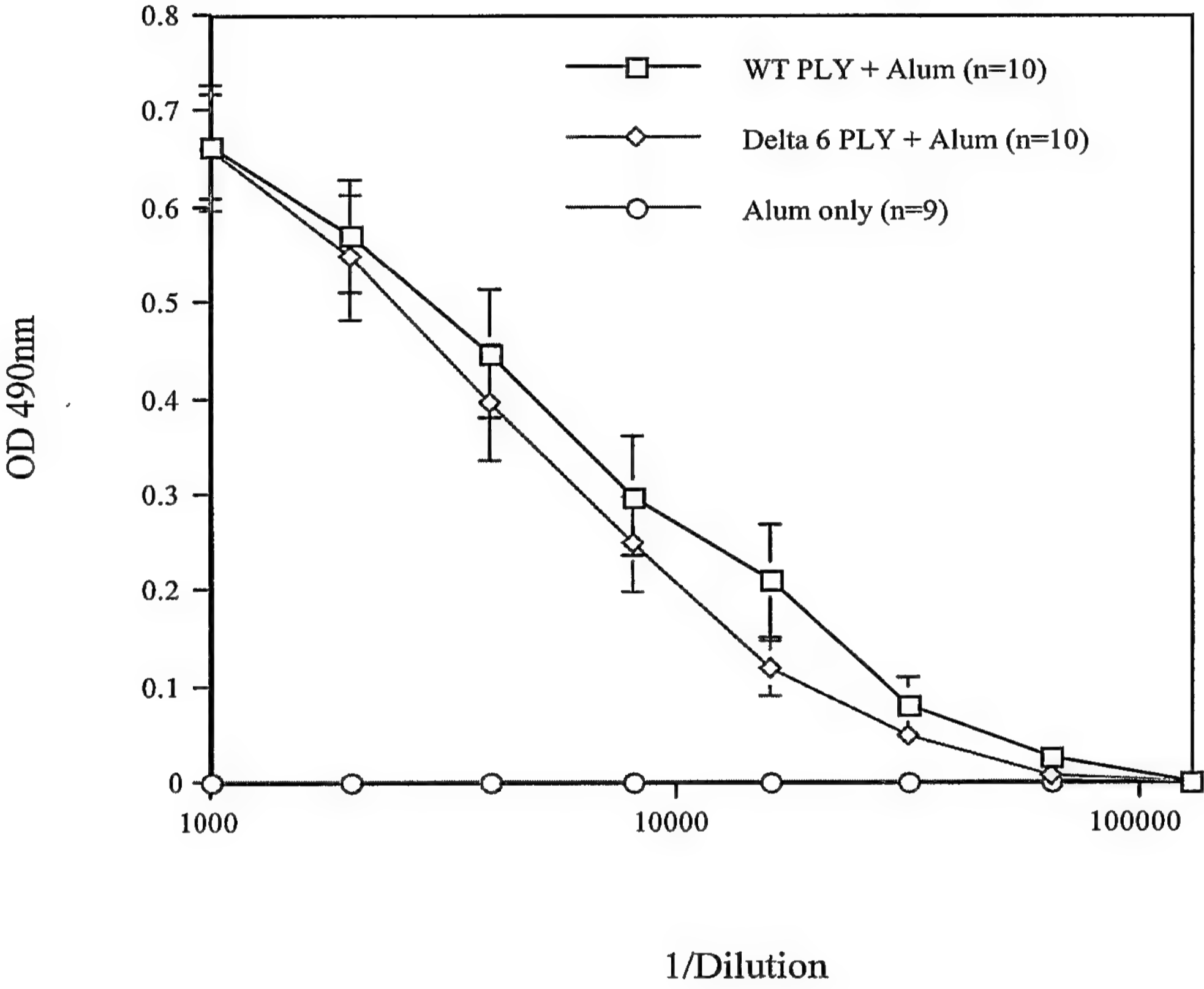


Figure 9

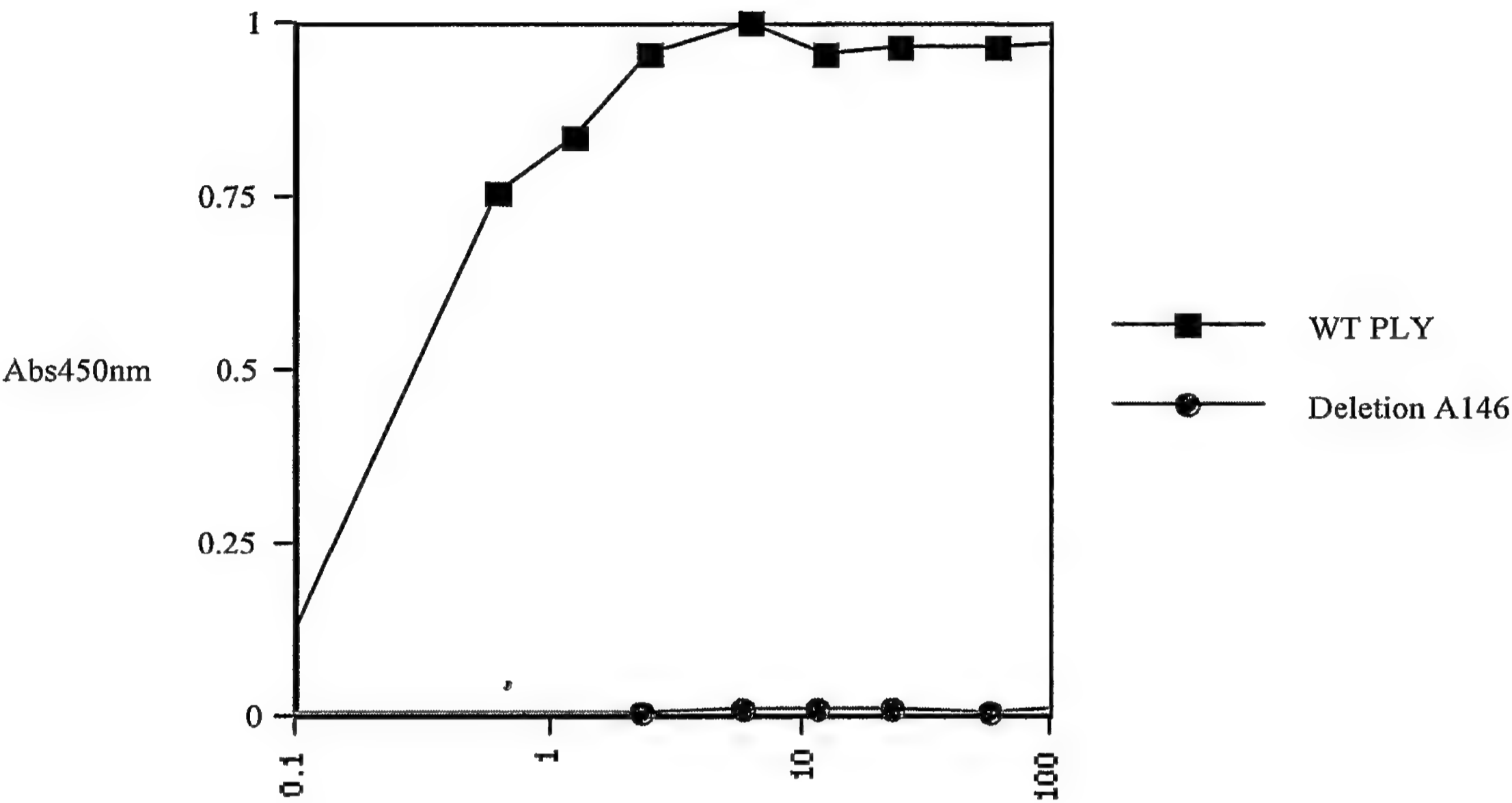


Figure 10

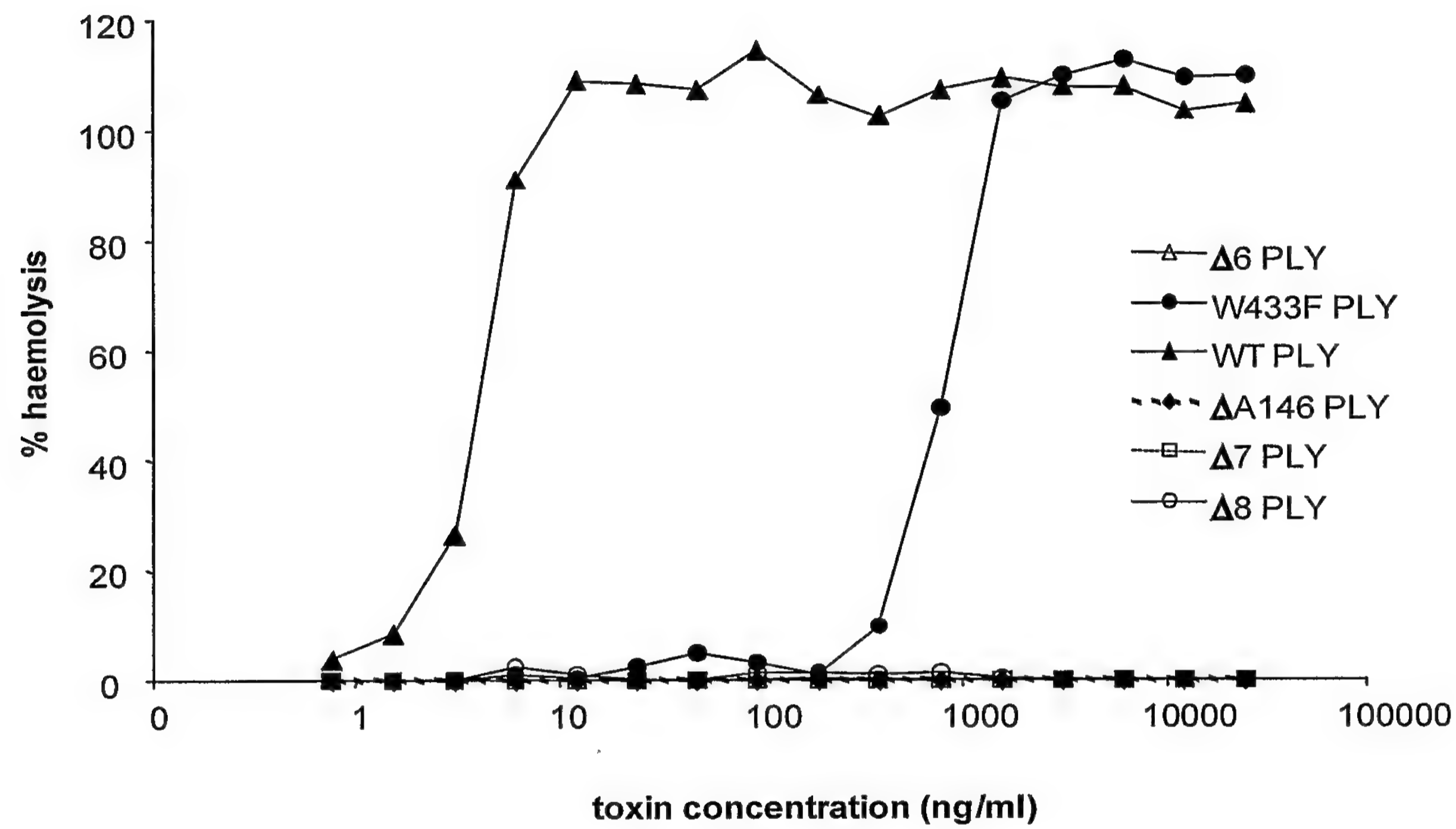


Figure 11

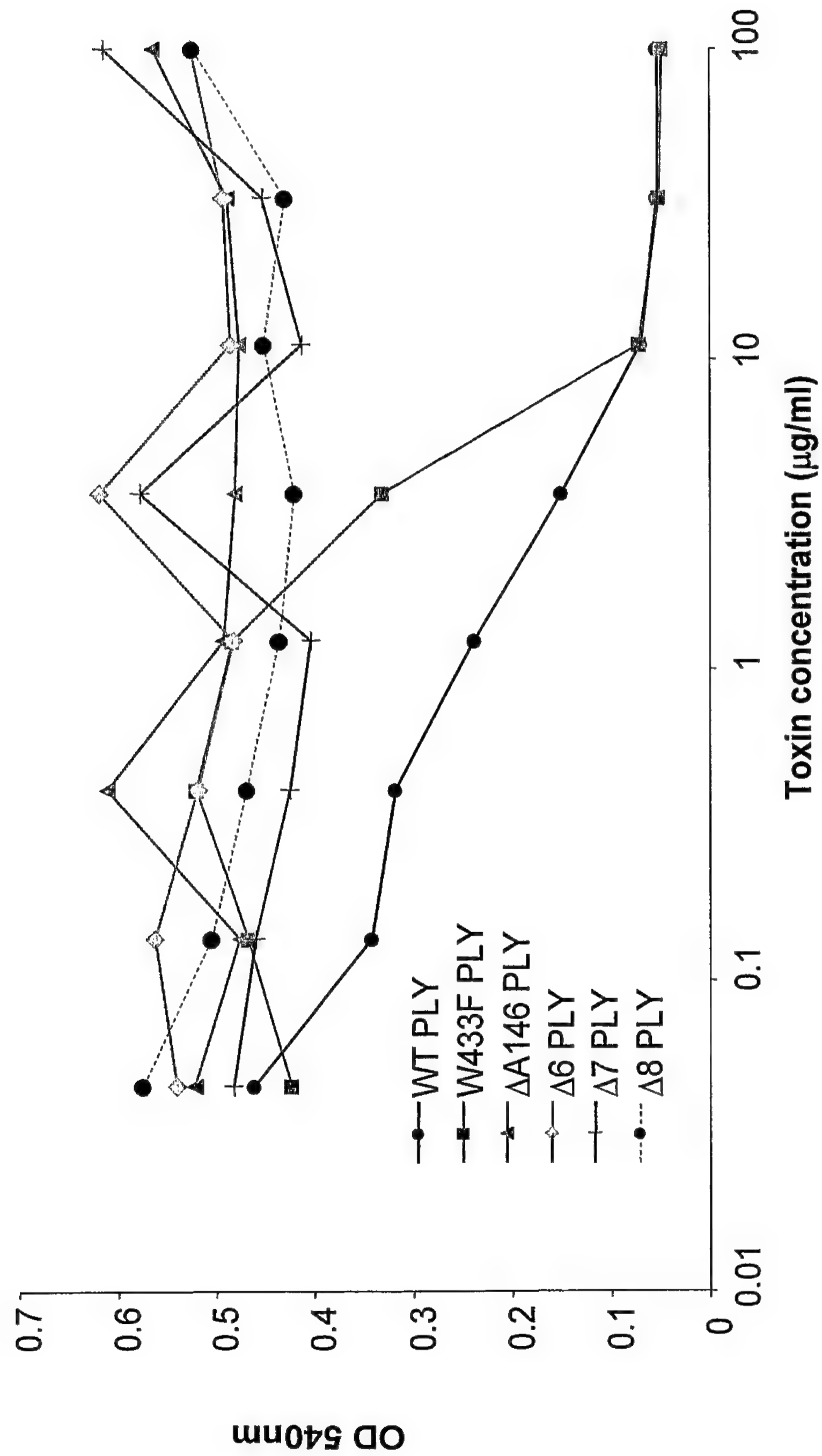


Figure 12

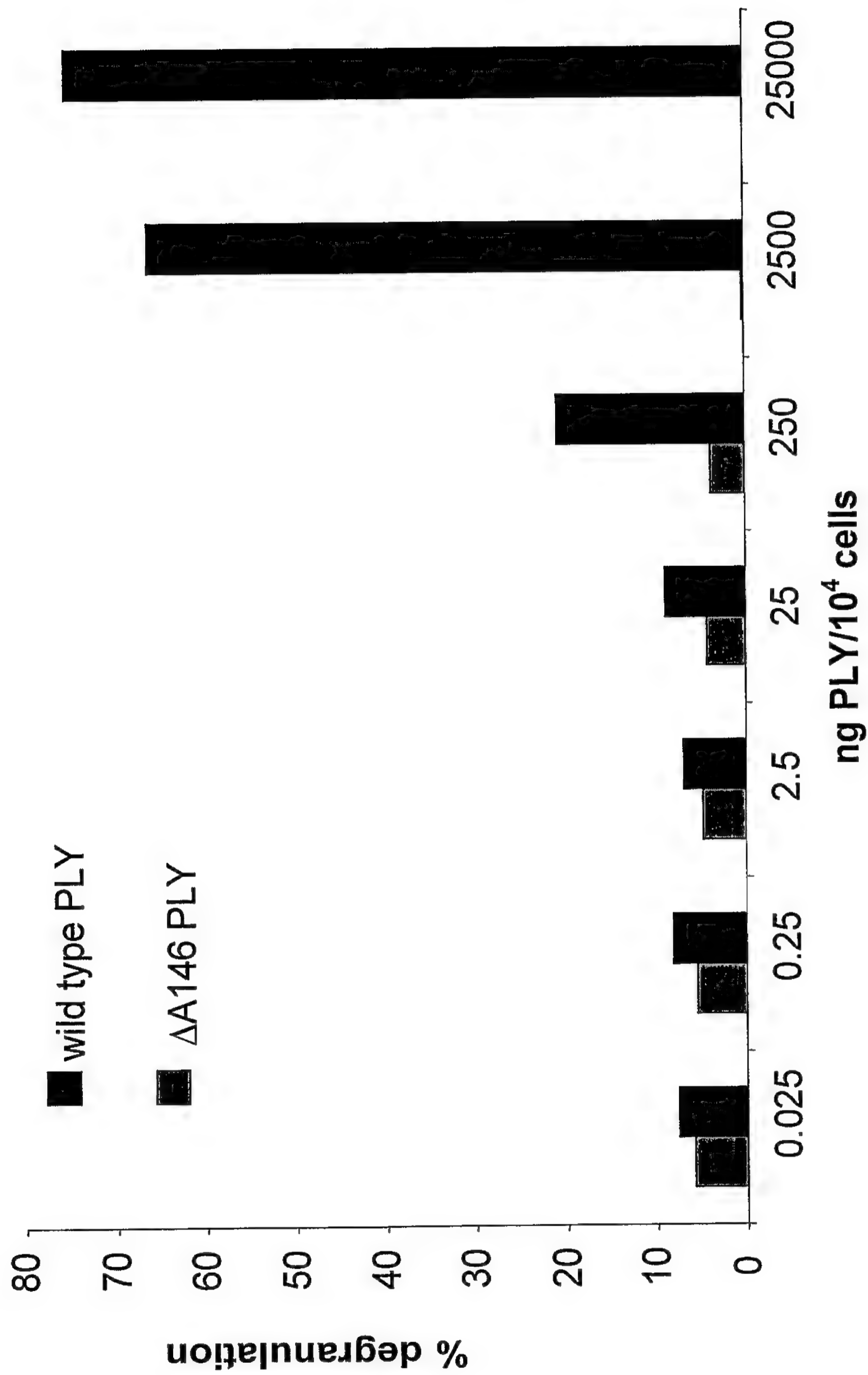


Figure 13

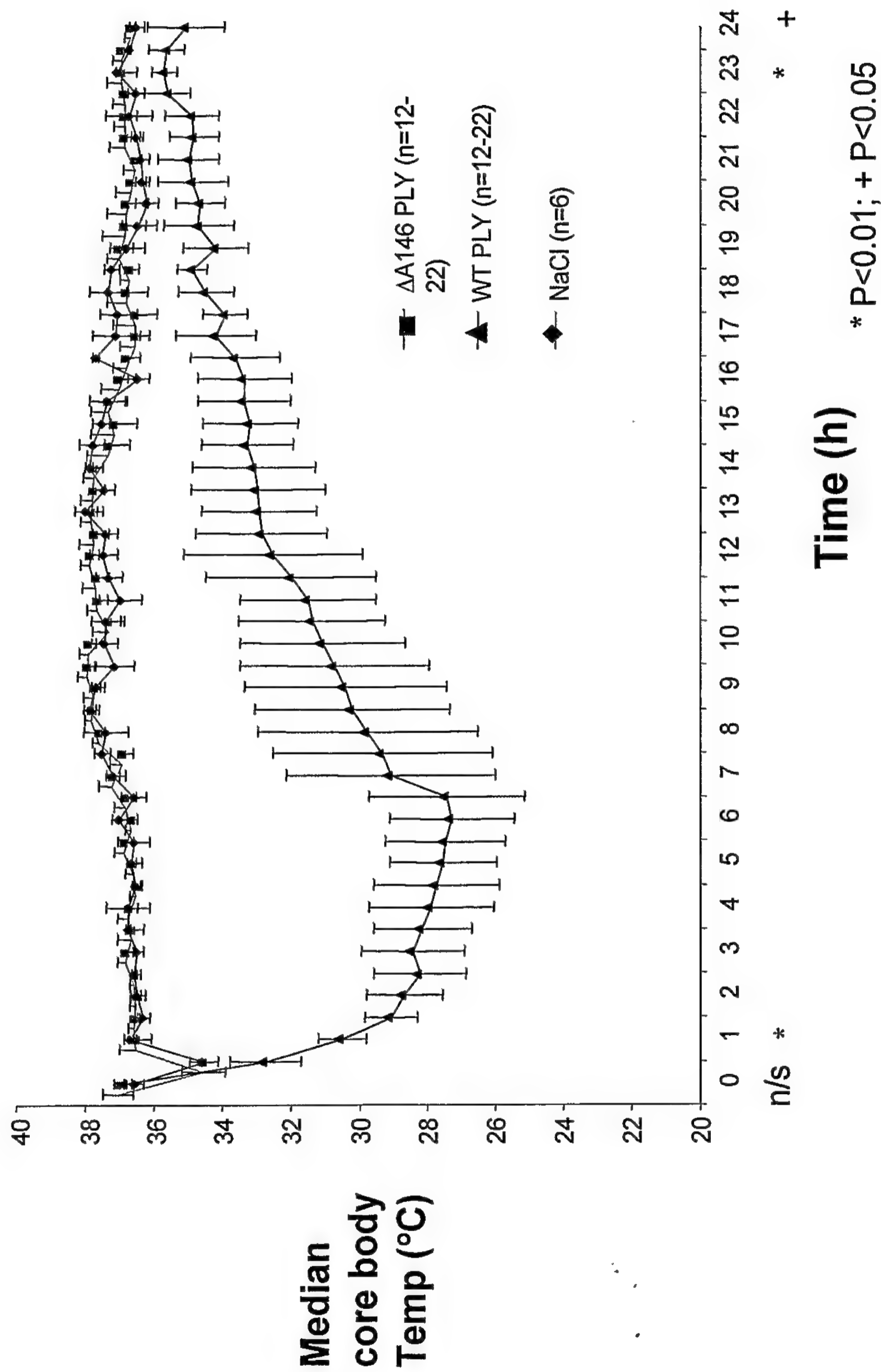


Figure 14

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2005/001792

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C07K14/315 A61K39/09 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE EMBL [Online] 21 May 1999 (1999-05-21), MINETTI C ET AL: "Streptococcus pneumoniae type 14 pneumolysin mutant" XP002336837 Database accession no. AAW97795</p>	<p>1-4, 11-15, 22-26, 28, 34-41, 48-52, 59-67 1-67</p>
Y	<p>abstract -& WO 99/03884 A (NORTH AMERICAN VACCINE, INC; MINETTI, CONCEICAO; MICHON, FRANCIS; PULL) 28 January 1999 (1999-01-28) claims; examples; tables 4,5 ----- -/--</p>	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

1 August 2005

Date of mailing of the international search report

13. 10. 2005

Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2005/001792

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 716 432 B1 (PATON JAMES CLELAND ET AL) 6 April 2004 (2004-04-06)	1,2, 11-13, 22-25, 34-39, 48-50, 59-67
Y	column 2, line 22 - column 3, line 45; claims; table 1	1-67
X	----- DATABASE EMBL [Online] 23 November 1990 (1990-11-23), PATON JC ET AL: "Immunogenic pneumolysin variant" XP002336836 Database accession no. AAR05924	1,2, 11-13, 22-25, 34-39, 48-50, 59-67
Y	abstract -& WO 90/06951 A (PATON, JAMES, CLELAND; HANSMAN, DAVID, JOHN; BOULNOIS, GRAHAM, JOHN; A) 28 June 1990 (1990-06-28) claims	1-67
X	----- HILL J ET AL: "Amino acids in pneumolysin important for hemolytic activity identified by random mutagenesis" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 62, no. 2, February 1994 (1994-02), pages 757-758, XP001194645 ISSN: 0019-9567 page 758, paragraph 1; table 1	1,2,24, 25, 35-39,65
P,X	----- KIRKHAM L S ET AL: "Construction and characterisation of a non-toxic form of pneumolysin for use as a vaccine carrier protein" IMMUNOLOGY, vol. 113, no. s1, 26 November 2004 (2004-11-26), page 2, XP002336832 abstract & KIRKHAM L S ET AL.: ANNUAL CONGRESS OF THE BRITISH SOCIETY FOR IMMUNOLOGY, 2004, 7-10 December, Harrowgate, UK	1-67
P,X	----- KIRKHAM L S ET AL.: "Creation and characterisation of a non-toxic pneumolysin mutant" 4TH INTERNATIONAL SYMPOSIUM ON PNEUMOCOCCI AND PNEUMOCOCCAL DISEASES, [Online] 9 May 2004 (2004-05-09), page 243, XP002336833 Helsinki, Finland Retrieved from the Internet: URL:www.congrex.fi/isppd-4/images/abstract .pdf> [retrieved on 2005-05-18] abstract	1-67

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2005/001792

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 62 and 63 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-67

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-67

an isolated mutant pneumolysin (PLY) protein, wherein the mutant PLY protein differs from the wild type PLY protein by the presence of a mutation within the region of amino acids 144 to 161 of the wild type sequence, such that the toxicity of the mutant is reduced relative to that of the wild type protein as well as subject-matter related thereto;

1.1. claims: 1-5,11-16,22-27,34-42,48-53,59-67 (all partially)

an isolated mutant pneumolysin (PLY) protein, wherein alanine 146 is substituted or deleted as well as subject-matter related thereto;

1.2. claims: 7,18,30,44,55 (all completely), 1-4,6,11-15,17,22-26,28, 29,34-41,43,48-52,54,59-67 (all partially)

an isolated mutant pneumolysin (PLY) protein, wherein amino acids valine 144 and proline 145 are deleted as well as subject-matter related thereto;

1.3. claims: 8,19,31,45,56 (all completely), 1-6,11-17,22-29,34-43, 48-54,59-67 (all partially)

an isolated mutant pneumolysin (PLY) protein, wherein amino acids alanine 146 and arginine 147 are deleted as well as subject-matter related thereto;

1.4. claims: 9,20,32,45,57 (all completely), 1-4,6,11-15,17,22-26,28, 29,34-41,43,48-52,54,59-67 (all partially)

an isolated mutant pneumolysin (PLY) protein, wherein amino acids methionine 148 and glutamine 149 are deleted as well as subject-matter related thereto;

1.5. claims: 10,21,33,47,58 (all completely), 1-4,6,11-15,17,22-26,28, 29,34-41,43,48-52,54,59-67 (all partially)

an isolated mutant pneumolysin (PLY) protein, wherein amino acids tyrosine 150 and glutamic acid 151 are deleted as well as subject-matter related thereto;

2. claim: 68

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

a method for screening candidate mutant PLY proteins for suitability for use in immunogenic compositions, the method comprising the steps of providing a mutant PLY protein; testing the mutant protein for haemolytic activity; testing the mutant PLY protein for oligomerisation activity and comparing the mutant PLY protein haemolytic and oligomerisation activity with those of a non-mutant protein as well as subject-matter related thereto;

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int Application No
PCT/GB2005/001792

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9903884	A	28-01-1999	AU 740956 B2	15-11-2001
			AU 8407898 A	10-02-1999
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			ES 2160570 T3	16-11-2001
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